COMPARATIVE ANALYSIS OF CELLULASE PRODUCTION BY TORULA THERMOPHILA AND HUMICOLA INSOLENS

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Approval of the Graduate of Natural and Applied Sciences

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as thesis for the degree of Master of Science.

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ABSTRACT

COMPARATIVE ANALYSIS OF CELLULASE PRODUCTION BY TORULA THERMOPHILA AND HUMICOLA INSOLENS

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The cellulase enzyme complex consists of three different enzymes, endoglucanase, exoglucanase and β-glucosidase which hydrolyse synergistically β-1,4-D-glucosidically linkages of cellulose and is produced by a variety of organisms including thermophilic fungi.

In this study, the cellulolytic activity and growth of two thermophilic fungi, the newly isolated strain of *Torula thermophila* and the industrial strain of *Humicola insolens*, were investigated. To optimize the enzyme assay and cultivation conditions, shake flask cultures were used with Avicel as the carbon source. The fungi produced higher endoglucanase and filter paper activities when grown in the modified YpSs medium. The optimum pH and temperature of the activity of endoglucanase were determined to be pH 6.0 and 65°C, and

pH 6.5 and 60°C for *T.thermophila* and *H.insolens*, respectively. Study on the effect of temperature on the cellulase production showed that both organisms grew and produced cellulases in the range of 35 to 55°C. A temperature range of 40-50°C seemed to favour growth and cellulase production, though slight changes in this range did not significantly alter the enzyme production. *T.thermophila* was found to be as good cellulase producer as the industrial strain of *H.insolens*, implying that this strain with further optimizations might be used in the industrial production of cellulases.

Keywords: *Torula thermophila*, *Humicola insolens*, Endoglucanase, Avicel-adsorbable Endoglucanase.

ÖZ

TORULA THERMOPHILA VE HUMICOLA INSOLENS SELÜLAZ ÜRETİMİNİN KARŞILAŞTIRMALI ANALİZİ

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Selülaz enzim sistemi üç farklı enzimden oluşmaktadır. Bunlar endoglukanaz, ekzoglukanaz ve β-glukosidaz olarak adlandırılıp, birbirine bağlı olarak selülozun β-1,4-D-glukosidik bağlarını parçalar ve termofilik küfler dahil birçok organizma tarafından üretilir.

Bu çalışmada, yeni izole edilmiş *Torula thermophila* suşunun ve endüstriyel *Humicola insolens* suşunun selülaz aktivitesi ve küflerin üremesi araştırılmıştır. Enzim tayin etme ve küflerin büyüme koşullarını optimize etmek için karbon kaynağı olarak Avicel ile çalkamalı kültürler kullanılmıştır. Küfler, YpSs ortamında daha yüksek endoglukanaz ve filtre kağıdı aktivitesi göstermişlerdir. Endoglukanaz aktivitesi için optimum pH ve sıcaklık *T.thermophila* ve *H.insolens* için sırasıyla pH 6.0 ve 65°C, pH 6.5 ve 60°C

olarak belirlenmiştir. Sıcaklığın selülaz üretimindeki olan etkisi ise her iki organizmanın 35–55°C aralığı içinde büyüdüğü ve selülaz ürettiğini göstermiştir. 40 ve 50°C arasındaki sıcaklarda selülaz üretiminin ve büyümenin arttığı görülmüştür, fakat bu ısı aralığında küçük değişimler enzim üretimini önemli derecede etkilememektedir. *T.thermophila*, *H.insolens* endüstriyel suşu kadar iyi selülaz üreticisi olarak bulunmuştur. Bu bağlamda, bu küfün optimize edilmesiyle endüstriyel selülaz üretiminde kulanılabilceği düşünülmektedir.

Anahtar Kelimeler: *Torula thermophila*, *Humicola insolens* Endoglukanaz, Avicel üzerine bağlanabilen endoglukanaz

TO MY FAMILY

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ABBREVIATIONS

AAEG	Avicel-Adsorbable Endoglucanase
CBD	Cellulose Binding Domain
CBH	Cellobiohydrolase
CMC	Carboxymethyl Cellulose
CMCase	Carboxymethyl Cellulase
EG	Endoglucanase
FPA	Filter Paper Activity
HEC	Hydroxyethyl Cellulose

CHAPTER 1

INTRODUCTION

1.1 Structure of Cellulose

In nature, cellulose is found in a complex with lignin and hemicellulose in the cell wall of plants. It is a linear, essentially insoluble, β-1,4-D-glucosidically linked polymer containing 8,000-14,000 glucose units forming a crystalline unit (Fig. 1.1). Although the chemical composition is simple, the physical structure and morphology of cellulose are complex. Hydrogen bonds and van der Waal's forces hold adjacent cellulose molecules together enabling their parallel alignment to produce a crystalline structure. Experimentally, it was determined that a cellulose crystal is formed by glucan chains in a parallel orientation with reducing chain ends at one terminus and non-reducing chain ends at the other. In fact, cellulose contains amorphous regions together with crystalline ones.

In native cellulose, the cellulose molecules are organized into fibers. In general, it is supposed that cellulose polymers are arrayed side-by-side in crystalline structures over 85 % of their length, and in more amorphous configurations over the remaining 15 %. Aggregates of these cellulose molecules form the elementary fibrils, which are attached side by side to each other to form the microfibrils. These microfibrils are embedded in a matrix of lignin and hemicellulose in order to obtain the rigid and inert wall structure of plant cells (Sharrock, 1988).

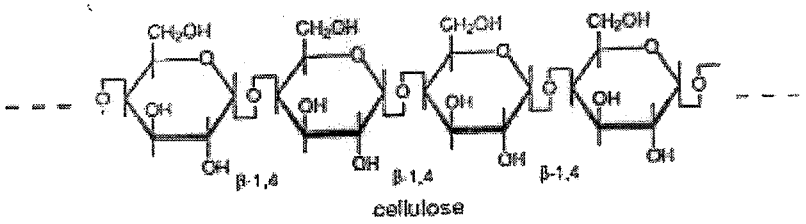


Fig. 1.1 Structure of cellulose

Because of the complex physical structure and morphology of cellulose, its hydrolysis is quite difficult, depending on the degree of crystallinity. Therefore, the enzymatic hydrolysis of cellulose requires many different enzymes.

1.2 Enzymatic Hydrolysis of Cellulose

The enzymatic hydrolysis of cellulose to glucose is performed by the cellulase enzyme complex which consists of endoglucanases, exoglucanases and β-glucosidases acting in a synergistic fashion. Cellulose is one of the world's most abundant organic polymers. It is found mainly in the cell wall of terrestrial plants and marine algae and is also synthesized by other organisms such as some bacteria. Actually, cellulose is an inedible biomass which can be converted into a valuable product by applying the proper hydrolysis. Cellulose hydrolysis might be achieved by chemical or enzymatic degradation. Nowadays, enzymatic hydrolysis gains much more significance in industrial applications because of the higher specificity of the reactions, better operational control and milder processing conditions.

1.2.1 Cellulase Enzyme Complex

Cellulase is an enzyme complex consisting of three general classes of hydrolytic enzymes:

1. Endocellulases (1,4-β-D-glucan-4-glucanohydrolase, endoglucanase, EG, E.C. 3.2.1.4) randomly cleaving β-1,4 glucosidic bonds on cellulose.
2. Exocellulases (1,4-β-D-glucan-4-cellobiohydrolase, exoglucanase, cellobiohydrolase, CBH, E.C. 3.2.1.91) releasing cellobiose units from the non-reducing ends of cellulose.
3. β-glucosidases (β-D-glucosido-glucohydrolase, cellobiase, E.C. 3.2.1.21) hydrolysing cellobiose to glucose.

1.2.2 General Features of Cellulases

The cellulase enzyme complex, which consists of at least three general classes of hydrolytic enzymes, is responsible for the degradation of cellulose. The fungal cellulase system, based on *Trichoderma* cellulases includes endoglucanases, cellobiohydrolases and β-glucosidases. These enzymes are classified with respect to an assumed substrate specificity. Since the cellulase system is not quite well understood and still under research, in the literature, there are different classifications of the cellulolytic enzymes. According to Henrissat *et al.* (1989), and Henrissat and Bairoch (1993), the fungal cellulases can be divided into five families (family 5, 6, 7, 12, and 45) with respect to the catalytically active core structures. Further, the cellulases of fungi can be classified into two groups: One group consisting of enzymes with a single-domain cellulolytic core, and the other group consisting of enzymes with two-domain structures, in which the catalytic domain is bound to the cellulose binding domain by a linker region.

Although many studies on cellulases and their mode of action have been done, yet, it is not clearly well understood how the components of the enzyme complex interact on the surface of crystalline cellulose and complete the extensive hydrolysis of cellulose. But the assumed classical action of the cellulase complex is as follows: EGs initially cleave internal cellulosic linkages, followed by the combined action of CBHs and EGs, and finally β-glucosidases hydrolyze cellobiose to glucose (Fig. 1.2).

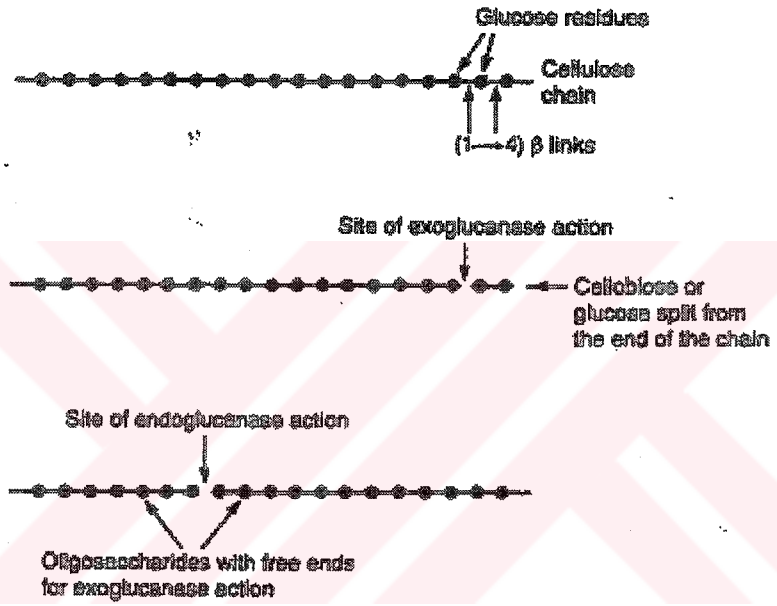


Fig. 1.2 Action of Cellulases onto Cellulose Chain

Studies on the action of cellulases indicate that synergism occurs between the individual components of the complex. Synergism was found to occur between EGs and CBHs, between CBH components and occasionally between EG and β-glucosidase components (since these enzymes occur in multiple forms). Also synergism was observed between cellulases of different

microorganisms (Goyal *et al.*, 1991). The synergistic interactions between the components of the cellulase complex were found to be most frequent when crystalline cellulose was the substrate and were low or absent when amorphous cellulose (H_3PO_4-swollen cellulose) or soluble derivatives of cellulose (CMC, HEC) were the substrates (Sharrock, 1988).

Most of the fungal cellulases are glycoproteins, consisting of a two-domain structure. The cellulose binding domain (CBD) and the catalytic domain are linked by a linker region which is usually composed of a short amino acid sequence with varying length (6-59), rich in proline and hydroxyamino acids often highly glycosylated. The CBDs of the cellulase enzymes are required to target the catalytic domain to cellulose in order to achieve efficient hydrolysis. Usually, the catalytic domains of cellulases are large and comprise more than 70 % of the total protein (Bhat and Bhat, 1997).

1.2.2.1 Endoglucanases

Endoglucanases (or Carboxymethyl cellulases, CMCases) have an important role in the hydrolysis of cellulose and nowadays, these components of the cellulase system find more use in various industrial applications. In general, EGs are defined as the enzymes that cleave randomly β-1,4-D-glucosidic linkages of the cellulose chain. Usually, these enzymes cannot degrade crystalline cellulose (e.g.: Avicel, Solca Floc), but can effectively hydrolyze substituted soluble cellulosic substrates (e.g.: CMC, HEC). Though many studies have been done, the exact role of EG in the degradation of native cellulose is not clearly understood.

Studies on the EGs of *Trichoderma viride* revealed that this fungus produced six different EGs, and carbohydrate staining showed that these enzymes are glycoproteins (Walker and Wilson, 1991). The studies on the EGs from different fungal species have shown that microorganisms may produce more than one EG, the EGs synthesized by a certain organism might differ in

activity, substrate specificity, amino acid sequence, molecular weight, isoelectric point, and so on. Therefore, a classification of the different EGs had to be done.

In the literature, the nomenclature and classification of EGs is confusing since, in general, the enzymes are numbered according to the time when they were first discovered. The International Enzyme Commission has recommended that such enzymes should be categorized according to their isoelectric points. Besides these, some researchers (Kyriacou *et al.*, 1987, Henrissat *et al.*, 1989, Schülein, 1997) have classified EGs into two groups: One group bearing a cellulose binding domain bound to the catalytic domain via a linker region (Fig. 1.3), and the other group lacking a cellulose binding domain (CBD). Due to the difference in the structure of these two types of EGs, they also differ in their activities towards different cellulosic substrates. EGs with CBDs can bind to crystalline cellulose and even some of them can degrade it, while the EGs without CBDs do not show any activity towards crystalline cellulose, but are active on soluble derivatives of cellulose.

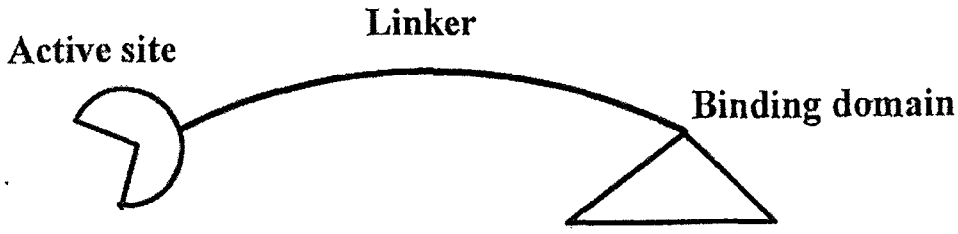


Fig. 1.3 Structure of Cellulase with a CBD

Kyriacou *et al.* (1987) have purified and characterized the cellulolytic enzymes of *Trichoderma reesei* RUT C-30. They have suggested that the EGs produced by *T.reesei* RUT C-30 might be categorized into two types; the specific EGs which act on the internal glucosidic bonds of cellulose chains only, and the non-specific EGs that are also capable of hydrolyzing xylans and crystalline cellulose.

Yoshioka *et al.* (1982) suggested that two types of EGs were synthesized by *Humicola grisea* var. *thermoidea* YH-78. They showed that *H.grisea* var. *thermoidea* produced Avicel-adsorbable EG that could disintegrate Avicel and the ordinary type of Avicel non-adsorbable EG active only on soluble cellulosic substrates.

A protease-negative mutant of *H.grisea* var. *thermoidea* YH-78 was obtained and the EGs produced by this mutant have been purified and characterized. The molecular weight of Avicel-adsorbable EG was 128,000, and that of Avicel non-adsorbable EG was 63,000. Avicel-adsorbable EG was stable in the pH range 3.0-11.0, while the optimum pH was 5.0. Also, this enzyme was stable to heating at 60°C for 5 min. and the optimum temperature was 50°C. Studies on the enzyme adsorbability showed that purified Avicel-adsorbable EG was adsorbed 72 % onto Avicel and 70 % onto cellulose powder. Besides high activity towards CMC, also it was observed that Avicel-adsorbable EG could completely disintegrate filter paper within 30 min. and Avicel within 24 hr at 50°C (Hayashida and Mo, 1986).

Hayashida *et al.* (1988) have observed the action of Avicel adsorbable EG from the protease-negative mutant of *H.grisea* var. *thermoidea* towards Avicel by scanning electron microscopy. Avicel-adsorbable EG disintegrated Avicel fibrils layer by layer from the surface, thus yielding thin sections with exposed chains. Studies on the synergistic action have revealed that Avicel-adsorbable EG showed a higher synergistic effect with CBH than with Avicel non-adsorbable EG, and it was suggested that Avicel-adsorbable EG contained 'cellulose-affinity site' which was different from the cellulase active site.

The mesophilic fungus *Humicola grisea* FB, isolated from forest soil, was found to produce Avicel-adsorbing EG. Adsorbability studies have shown that 22 % of the EG was adsorbed onto Avicel (Soundar and Chandra, 1988).

Humicola spp. H_1 was found to produce Avicel-adsorbable EG when grown in culture medium with 1 % water hyacinth as carbon source. It was observed that 13 % of the EG present in the filtrate was adsorbed onto Avicel and that the cellulases of *Humicola* spp. H_1 were stable up to 70°C (Ali *et al.*, 1993).

Schülein (1997) has purified and characterized five EGs from *Humicola insolens.* EG I and EG III do not possess CBD, while EG II, EG V, and EG VI have CBDs. All the EGs showed maximum activity in the pH range of 7.0 to 8.5. While EG I, EG II and EG III exhibit similar activities towards CMC and amorphous cellulose; EG V and EG VI have the highest catalytic rate on amorphous cellulose. EG I belongs to family 7 cellulases, being constructed of 415 amino acid residues, the 398 of which forming an ordered structure, while the other 17 amino acids not required for catalytic activity. EG II belongs to family 5 glycosyl hydrolases with the N-terminal CBD. Studies on the amino acid sequence have revealed that the amino acid sequence of EG II of *H.insolens* bears 50 % homology with EG II from *T.reesei.* EG III is a family 12 EG and is made of 224 amino acid residues. *Trichoderma* and *Aspergillus* sp. can also produce such small cellulases. EG V belongs to family 45 cellulase with a CBD at the C-terminal. The structure of EG V was determined by Davies *et al.* (1995) (See: Sec. 1.2.1). EG VI is a family 6 EG with a CBD at the C-terminal.

1.2.2.2 Exoglucanases

Exoglucanases (or cellobiohydrolases, CBHs) are the components of the cellulase complex which hydrolyze 1,4-β-D-glucosidic linkages in cellulose, releasing cellobiose units from the non-reducing ends of the cellulose chain.

They are active on swollen, partially degraded, amorphous cellulose and show limited or no activity on soluble derivatives of cellulose.

The CBHs of *T.reesei* have been studied in detail and it was found out that this fungus produced two distinct cellobiohydrolases, CBH I and CBH II. Both of these enzymes were glycoproteins with different amino acid composition and showing different activities towards different types of cellulose. CBH I was the major enzyme comprising 60 % of the secreted proteins. Purified CBH I from several sources had molecular weights ranging from 42 to 72 kDa with carbohydrate content from 1.4 to 10.4 % and isoelectric points from 3.5 to 4.2. The amino acid sequence of CBH I was determined and found to consist of 496 amino acids. CBH I had 12 disulfide bridges and thus, there were no free cysteine residues (Goyal *et. al.*, 1991).

CBH II of *T.reesei* constituted around 20 % of the secreted proteins. The molecular weights of CBH II from different sources ranged from 50 to 58 kDa, while the isoelectric points ranged from 5.0 to 6.3 and these enzymes show 8-18 % glycosylation (Goyal *et al.*, 1991). Also, it was observed that CBH II exhibited low activity towards CMC and caused short fiber formation besides its activity towards amorphous cellulose. CBH II more closely resembled an EG than a cellobiohydrolase such as CBH I (Kyriacou *et al.*, 1987).

Besides the CBHs of *T.reesei*, those of *H.insolens* have also been investigated. Schülein (1997) had characterized the CBH I and CBH II of *H.insolens*. The pH activity profiles were determined, and CBH I and CBH II showed maximum activity at pH 5.5 and 9.0, respectively. CBH I was defined as a family 7 cellulase and it could also degrade crystalline cellulose as well as amorphous cellulose. The number of amino acid residues of CBH I was determined for core region, linker region and CBD as 437, 42, and 36, respectively. CBH II was a family 6 cellulase with N-terminal CBD. The sequence of CBH II of *H.insolens* exhibited 65 % homology with the CBH II of *T.reesei.* The number of amino acid residues of CBH II from *H.insolens* were determined, and for core region, linker region and CBD were 366, 45, and 38, respectively (Schülein, 1997).

The CBH I of *Humicola grisea* var. *thermoidea* was cloned and expressed in *Aspergillus oryzea*, and the gene products were purified. The *H.grisea* CBH I showed 37.7 % identity with *T.reesei* EG. The optimal temperature and pH for CBH I were 60°C and 5.0, respectively. CBH I showed high activity towards Avicel (Takashima *et al.*, 1996).

1.2.2.3 β-Glucosidases

β-Glucosidases (or cellobiases) are hydrolases which act on β-linked diglucosides and aryl-β-glucosides. Though β-glucosidases do not act on cellulose, they are regarded as components of the cellulase enzyme system because they have been found to stimulate the rate and extent of cellulose hydrolysis. They were found to relieve the inhibition of cellulases by cellobiose formed from the action of EGs and CBHs.

β-glucosidase from *Sporotrichum thermophile* has been purified and characterized. It was found to exhibit maximum activity at pH 5.4 and 65°C. At 50°C, *S.thermophile* β-glucosidase was stable for at least 6 hr at pH 4.0-6.5. The molecular mass of the enzyme was found to be 240 kDa. Also, a single 110 kDa protein was found, thus, showing that the enzyme was composed of two similar subunits. Studies on the time of β-glucosidase production showed that very low cellobiase was produced during active growth, suggesting that β-glucosidases were the major proteins which were released during autolysis in *S.thermophile.* Thus, the maximal activity of β-glucosidase from *S.thermophile* was obtained during idiophase. Study on the inhibition of β-glucosidase showed that like the cellobiases of other microorganisms, that of *S.thermohile* was also inhibited by glucose (Bhat *et al.*, 1993).

Hayashida *et al.* (1988) have characterized β-glucosidase from *H.insolens* YH-8. The molecular weight was determined to be 250,000 and the isoelectric point was 4.23. The carbohydrate content of β-glucosidase was

estimated to be 2.5 %, and the constituent sugars were 1.7 % mannose and 0.8% glucose. The study on the amino acid composition showed that aspartic acid, glycine and alanine were abundant.

β-glucosidase from solid-state cultures of *Humicola grisea* var. *thermoidea* has been purified and characterized. Two proteins with molecular mass of 82 and 156 kDa have been purified, indicating that the enzyme may consist of two subunits. The enzyme showed maximum activity at pH 4.0-4.5 and 60°C, and was very active against cellobiose and p-nitrophenyl β-D-glucopyranoside. *H.grisea* var. *thermoidea* β-glucosidase was stable at 60°C for 1 hr with a half-life of 15 min. at 65°C (Ferreira, 1996).

Peralta *et al.* (1997) have characterized a highly thermostable β-glucosidase from a strain of *H.grisea* var. *thermoidea*. The purified enzyme was a glycoprotein containing 35 % carbohydrate with molecular mass of 55 kDa. The optimum temperature and pH for enzyme activity was 50-60°C and 6.0, respectively. The enzyme was stable at 60°C and showed a half-life of 30 min. at 70°C.

1.3 Applications of Cellulases

Cellulolytic enzymes carry a great potential for applications in various industries. Since cellulose is one of the world's most abundant naturally produced organic polymer, annually great quantities of it are produced and also wastes containing cellulose are generated. By the use of cellulase enzymes, the inedible biomass of cellulose can be converted into valuable products, such as glucose, soluble sugars, alcohols, single cell protein and so on.

Nowadays, cellulolytic enzymes are used in food, feed, textile, detergent, medical/pharmaceutical, pulp and paper industries, and genetic studies.

In food industry, cellulolytic enzymes find applications in starch processing, grain alcohol fermentation, malting and brewing, beverage

production (clearing of fruit and vegetable juices), peeling of fruits, fruit candying, baking, degumming coffee extracts, extraction of agar from seaweeds, gelatinization of seaweeds, isolation of proteins from soybean and coconut and so on (Esterbauer *et al.*, 1991, Bhat and Bhat, 1997).

Besides food industry, cellulases are also important for animal feed production. In silage production, cellulolytic enzymes are used in order to obtain better hydrolysis of grain for monogastric animals, and thus, increase the digestibility of animal feed (Pentillä *et al.*,1991).

In biotechnological processes, cellulases are applied to lignocellulosics and saccharification might be achieved. Further, the product which is formed might be used in fermentation processes (e.g.: production of antibiotics ...). In addition, pure preparations of cellulases are used in genetic engineering studies for microbial/plant cell wall destruction and protoplast formation (Esterbauer *et al.*, 1991).

In detergent industry, cellulases are used in combination with lipases and proteases for color brightening, softening and particulate soil removal. For this purpose, only one or a specific combination of cellulases is required (Bazin and Sasserod, 1991).

In pulp and paper production, again a specific combination of the components of the enzyme complex should be applied to wood in order to avoid saccharification. Cellulases are used in paper industry for acceleration of pulping or for upgrading the pulp properties (Rho *et al.*, 1982).

Cellulases are intended to be used in fuel industry, but this has not yet been used on an industrial scale, because of the high cost of cellulase production. In laboratory conditions, it was established that cellulases could be readily used for the conversion of lignocellulosic wastes to liquid fuels such as ethanol. In the future, if this process could be made feasible, it would be a great achievement, because of the fuel oil shortages and environmental impact of petroleum based fuels (Chen and Wayman, 1991). In general, enzymatic treatments are considered to be much more environmentally friendly than

system in order to achieve the desired final product. Enzymatic treatment of textile fabrics will have several advantages when compared to chemical or physical treatments. These are: strongly reduced tendency for pilling formation, clearer surface structure with less fuzz, improved water absorbancy, and better process control (Bazin and Sasserod, 1991). Besides this, enzymatic treatment is much more environmentally friendly, since in stone washing of blue jeans, a dust is formed which can be dangerous for the workers in the factory, while with cellulase treatment the process is much easier and safer.

1.4 Microorganisms Producing Cellulase Enzymes

Many different microorganisms have been screened for cellulolytic activities. It has been found out that cellulases are produced by a wide variety of bacterial and fungal species, however, nowadays, fungi are emerging as better cellulase producers. Among the studied microorganisms, relatively few organisms produce the necessary group and level of cellulolytic enzymes required for the solubilization of crystalline cellulose.

Most of the studies on cellulases have been done on fungi, since this diverse group of organisms is an excellent source for extracellular enzymes. Fungi belonging to the *Ascomycetes, Deuteromycetes,* and *Basidiomycetes* classes are producing cellulolytic enzymes. The white rot fungi have the ability

to utilize lignocellulosic materials, thus producing ligninases as well as cellulases. The most studied white rot fungus, *Phanerochaete crysosporium* has been found to produce five endoglucanases, one exoglucanase and two β-glucosidases. Brown rot fungi (e.g.: *Tyromyces palustris, Poria placenta, Poria carbonica*) are also known to utilize cellulose but they differ from white rot and soft rot fungi in producing only endoglucanases. Soft rot fungi (e.g.: *Trichoderma* sp., *Aspergillus* sp., *Fusarium* sp., *Humicola* sp.) are capable of degrading lignocellulosic substrates, but they preferably utilize cellulosic materials. The most studied soft rot *Deuteromycete*, *Trichoderma reesei* has been found to produce at least six endoglucanases, three exoglucanases and one β-glucosidase acting synergistically in the degradation of cellulose to glucose (Goyal *et al.*, 1991).

Since cellulases have been discovered , studies have been done mainly on the aerobic fungi from *Trichoderma, Aspergillus, Penicillium, Phanerochaete* and *Fusarium* genera. Nowadays, species of these genera (*Trichoderma viride, T.reesei, Penicillium pinophilum, Aspergillus niger, Fusarium oxysporium*) appear to be the most powerful cellulase producers being used in the industrial production of cellulolytic enzymes. Besides these, there are many other mesophilic aerobic fungi capable of producing considerable amounts of cellulolytic enzymes (e.g.: *Talaromyces emersonii, Aspergillus terreus, Trichoderma koningi, Fusarium solani, Penicillium purpurogenum, Schyzophyllum commune*).

Recently, it has been found out that thermophilic aerobic fungi are also capable of producing cellulase enzymes (e.g.: *Sporotrichum* sp., *Humicola* sp., *Thermoascus aurantiacus*). Interest in the cellulolytic enzymes of these organisms increases because of their enzymatic properties and advantages of their production and cultivation processes.

Besides aerobic fungi, anaerobic fungi have also been investigated for cellulolytic activities. The most studied microorganisms are *Neocallimastix frontalis, Piromonas communis, Sphaeromonas communis.* Studies on the cellulase enzyme complex of *N.frontalis* have shown that it is a

multicomponent enzyme complex being different from that of aerobic fungi and termed as crystalline cellulose solubilizing factor (CCSF). The presence of the multicomponent enzyme complex of this fungus suggests that it may hydrolyze the crystalline cellulose using a mechanism which is similar to that of the anaerobic cellulolytic bacterium *Clostridium thermocellum* (Bhat and Bhat, 1997).

Bacteria producing cellulases may be either aerobic or anaerobic. Among the cellulolytic anaerobic bacteria *Clostridium thermocellum* is the most widely studied. Others include *Acetivibrio cellulolyticus, Bacteroides cellulosolvens, Ruminococcus albus, Ruminococcus flavefaciens*. *C.thermocellum* produces a high molecular mass enzyme complex called cellulosome, consisting of several endoglucanases and at least three exoglucanases, which degrade crystalline cellulose extensively.

Besides anaerobic bacteria, aerobic bacteria also produce cellulase enzymes (e.g.: *Cellulomonas* sp., *Celvibrio* sp.). *Acidothermus cellulolyticus,* which is a thermophilic, acidophilic, cellulolytic, aerobic bacterium has been found to produce filter paper cellulose degrading enzymes with extremely high thermostability, but with low enzyme activity (Shiang *et al.,* 1991).

Actinomycetes are also involved in lignocellulose degradation. In general, their cellulase system resembles that of fungal species. *Thermomonospora* sp., *Thermoactinomyces* sp., *Microbispora bispora, Streptomyces* sp. appear to be active cellulase producers.

1.5 Thermophilic Fungi as Cellulase Producers

Since the discovery of cellulose degrading enzymes a lot of research has been done on the potential cellulase producers, fermentation conditions, enzymatic properties, genetic features and industrial production. As the range of applications of cellulase enzymes broaden in various areas, the desired properties of these enzymes also change and nowadays, there is a world-wide

interest in enzymes which are stable at severe conditions (e.g.: high temperature, alkaline or acidic pH).

Only recently, it has been found out that thermophilic microorganisms are also capable of producing good yields of cellulases. Besides high yields and productivities, also the properties of the enzymes are taken into account, while considering the industrial applications. Usually, enzymes from thermophilic organisms are supposed to be more thermostable from those produced by mesophilic microorganisms. In addition, the cellulases from thermophilic fungi are more resistant to proteolysis and more stable to mechanical denaturation than those produced by mesophiles. Therefore, nowadays, there is a trend through studying cellulases from thermophilic fungal species. Besides enzymatic properties, the fermentation conditions of thermophilic fungi are also more advantageous than those for mesophilic ones. The higher temperatures used during cultivation of thermophilic microorganisms reduce the risk of contamination by other undesired organisms. Since on industrial scale of enzyme production, sterilization is not an economic process, the higher temperatures used during fermentation is an advantage. Although the fermentation costs might increase because of the higher energy requirements for cultivating thermophilic fungi, these microorganisms are preferred because much more aseptic conditions might be achieved and more thermostable products might be obtained. Therefore, the use of thermophilic cellulolytic fungi, with efficient thermostable enzymes, high rates of hydrolysis and ability to saccharify (act) under non-aseptic conditions, might make the hydrolysis of cellulose more economic (Latif *et al.*, 1995).

Considering the advantages of using thermophilic microorganisms for cellulase production, recently many studies have been done on cellulolytic thermophilic fungi. Some of these fungi are *Sporotrichum thermophile*, *Thermoascus aurantiacus*, *Humicola insolens*, *Malbranchea pulchella*, *Mucor pusillus*, *Chaetomium thermophile*, *Humicola grisea* var. *thermoidea*, *Thielavia terrestris*.

The thermophilic fungus, *Thermoascus aurantiacus* A-131, isolated from a soil sample has been found to produce constitutively thermostable cellulases in submerged fermentation conditions. Besides constitutive enzyme production, the cellulase production was induced markedly by amorphous polysaccharides containing β-1,4-linkages such as alkali-treated bagasse and xylan rather than crystalline cellulose. While comparing the thermostability of CMCase, Avicelase and β-glucosidase of *T.aurantiacus* A-131 with those of *Trichoderma reesei* QM 9414, it has been shown that the cellulase enzymes of *T.aurantiacus* A-131 are much more thermostable than the enzymes of *T.reesei* QM 9414. Also, it has been found that the CMCase productivity of *T. aurantiacus* A-131 is as high as that of *T.reesei* QM 9414 and higher than that of *Thielavia terrestris* (Kawamori *et al.*, 1987).

Latif *et al.* (1995) have been studied the production of cellulases by several thermophilic fungi including *Sporotrichum thermophile, Chaetomium thermophile, Humicola grisea, Torula thermophila, Malbranchea pulchella,* and *Mucor pusillus.* These fungi were grown on kallar grass straw as a carbon source, in liquid fermentation at 45°C and 120 rpm. While *S.thermophile* produced the highest β-glucosidase activity, *H.grisea* and *T.thermophila* showed moderate activities of FPA, CMCase and β-glucosidase, and *Ma.pulchella* and *Mu.pusillus* — low activities. But all microorganisms had the ability to produce the cellulase enzyme complex when grown on cheap lignocellulosic substrates, though with low productivity. The fact that these fungi are able to utilize raw lignocellulosic substrates demonstrated their potential for large-scale, low-cost use (Latif *et al.*, 1995).

A study on the production of endoglucanases from a protease-negative *Humicola grisea* var. *thermoidea* YH-78 mutant has shown that this thermophilic fungus produced two kinds of EGs: Avicel-adsorbable EG and Avicel unadsorbable EG, when the microorganism was grown in mold bran culture at 50°C (Hayashida and Mo, 1986).

H.grisea var. *thermoidea* strain IFO9854 was found to produce six β-glucosidases, two endoglucanases and one exoglucanase. The molecular weights of the purified enzymes ranged from 38,500 to 115,000 and some of the β-glucosidases were composed of several polypeptides. The optimum pH and temperature values for these enzymes were in the ranges 5.0-7.0 and 50-70°C, respectively (Takashima *et al.*, 1996).

Sporotrichum thermophile has been studied with respect to growth, cellulose degradation and cellulase activity. It has been found out that this thermophilic fungus produced all the major enzymes of the cellulase complex, but compared with the enzymes of *T.reesei* their activities were markedly lower. Although the enzyme activities were lower, *S.thermophile* degraded cellulose faster than *T.reesei*, when *S.thermophile* was grown at 50°C on 1 % alkali-treated blotting paper. Besides these, studies on the cellulolytic rates of some thermophilic fungi such as *Chaetomium thermophile* and *Thermoascus aurantiacus* have revealed that the cellulolytic rates of these fungi were two or three times that of *Trichoderma viride*, which is one of the most powerful cellulolytic mesophilic fungi (Bhat and Maheshwari, 1987).

1.5.1 *Humicola insolens*

Humicola insolens, is a thermophilic soft rot fungus belonging to subdivision *Deuteromycetes*, class *Hyphomycetes*, order *Moniliales*. *H.insolens* is a well-studied microorganism and nowadays some of its strains are used in the industrial production of cellulases for biopolishing of textiles. In nature, *H.insolens* grows readily in self-heating heaps, manure, mushroom composts, guayule pets (Cooney and Emerson, 1964). Also *H.insolens* is known to dominate toward the end of the second phase of fermentation of mushroom compost and can readily utilize lignocellulosic materials (Bilay and Lelley, 1997).

The thermophilic genus *Humicola* was studied in detail and was suggested as an excellent genus for production of extracellular enzymes. Besides production of cellulases and xylanases by *H.insolens* and *H.grisea* var. *thermoidea* strains, also lipases, glucoamylases and α-amylases are produced by *H.lanuginosa,* and amylases and trehalases in relatively high amounts – by *H.grisea* var. *thermoidea* (Campos and Felix, 1995).

Schülein (1997) has purified and characterized seven cellulases from a strain of *H.insolens* and found out that five of these cellulases are EGs and two CBHs. It was determined that the EGs of *H.insolens* can be divided into two groups: EGs having a cellulose binding domain (CBD) and EGs not having a CBD. EG I and EG III of *H.insolens* do not possess a CBD, while EG II, EG IV, EG V, and EG VI have a CBD. The kinetic constants for these enzymes have been determined and it was concluded that the presence of a CBD lowered the apparent K_m. This was due to the dispersion action of CBDs. Also, it was found that the CBD reduced the apparent k_{cat}, and the reason for this might be that the CBD slowed down the mobility. From this study, it became obvious that *H.insolens* possessed a battery of cellulose degrading enzymes which act synergistically to hydrolyze glucose efficiently (Schülein, 1997).

A study on the EG V of *H.insolens* has revealed that this enzyme is a glycoprotein consisting of 284 amino acids. The crystal structures of this enzyme, in native and oligosaccharide-bound forms had been determined at resolutions of 1.9 Å. It was observed that EG V was constructed of three distinct regions: a catalytic core domain, a linker region and a cellulose binding domain (CBD). The catalytic core domain consisited of 210 amino acids and was linked, *via* a heavily glycosylated serine and threonine rich linker region to the CBD. A comparison of the CBD of EG V from *H.insolens* with the CBD of CBH I from *T.reesei* had revealed high sequence identity. Besides this, it was observed that EG V consisted of a six-stranded β-barrel domain with a number of disulfide-bonded interconnecting loops and that it had six kinetically distinct subsites for carbohydrate binding. Also, it was determined by site-directed

mutagenesis that Asp10 and Asp121 were involved in the active site of EG V (Davies *et al.*, 1995).

H.insolens YH-8, isolated from manure, was found to produce thermostable CBH, EG, and β-glucosidase, with estimated molecular weights as 72,000, 57,000, 250,000 respectively by SDS-gel electrophoresis. Study on the relation of carbohydrate content and the thermostability of CBH and EG had indicated that there was relation between the thermal stabilities of the enzymes with their carbohydrate moieties, since when 90 % of the carbohydrate residues of CBH and EG was liberated, CBH lost its activity after heating at 75°C for 5 min. and EG – after heating at 75°C for 5 min. At normal carbohydrate content (in native form), EG retained 45 % of its original activity after heating at 95°C for 5 min. (Hayashida *et al.*, 1988).

A study on the effects of β-mercaptoethanol and sodium dodecyl sulfate on the β-glucosidase from *H.insolens*, showed that this enzyme was resistant to the effects of these strong denaturants (Rao and Murthy, 1991).

1.5.2 *Torula thermophila*

Torula thermophila like *H.insolens* is a thermophilic fungus belonging to subdivision *Fungi Imperfecti (Deuteromycetes),* class *Hyphomycetes,* order *Moniliales.* There is limited research on this organism, except taxonomical studies. *T.thermophila* can grow within a wider temperature range compared with other thermophiles such as *Chaetomium thermophile* and *Humicola stellata.* It is able to grow in the range of 23-58°C (Cooney and Emerson, 1964).

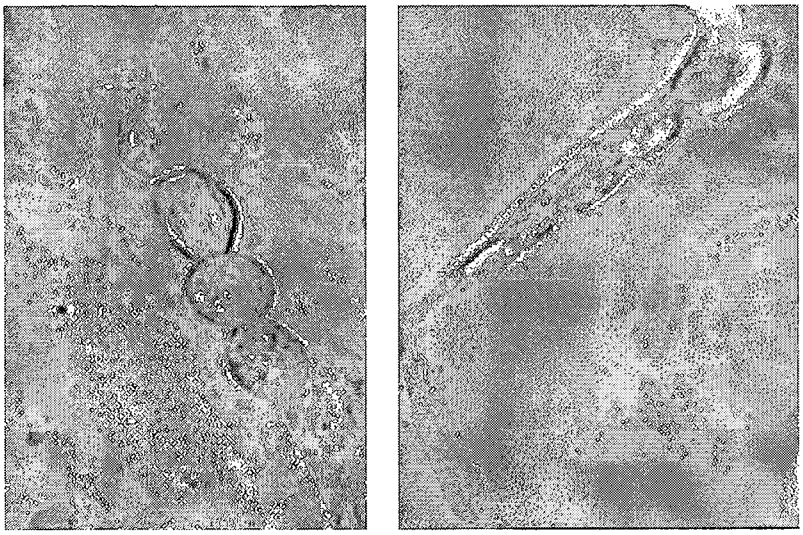
Three stains of *T.thermophila* have been isolated in our department. Samples from mushroom compost were taken and analyzed for the presence of thermophilic organisms. As a result of a number of subculturings, three fungal strains were isolated. The examination of these strains grown on YpSs agar

(given in Sec. 2.2.1), by the use of image analysis techniques, and further identification studies has revealed that all the isolates were *Torula thermophila*. It was observed that, in the early stages of sporulation, the hyphae were divided by segments called septa. When the spores became mature, they attained a spherical shape, and were either single or more commonly, in chains (Fig. 1.4 (a), (b) and (c)). After all mycelia turned into spores, the mycelia had the appearance of a 'soot' and the color was black (Tütek and Ceylan, 1996, Ögel *et al.*, 1998). These morphological characteristics were the same as those described for *T.thermophila* (Cooney and Emerson, 1964).

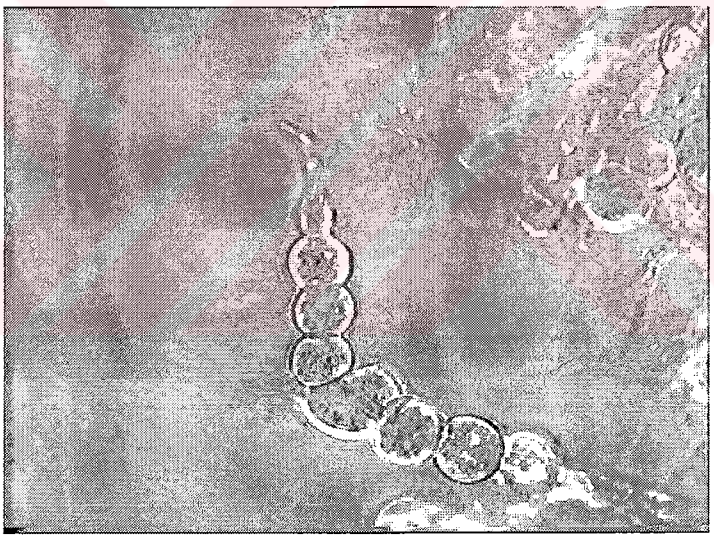
While comparing the spores of *Humicola* spp. (Fig. 1.5) with those of *T.thermophila* (Fig. 1.4) it was observed that the spores of *Humicola* spp. developed on short, lateral branches, whereas the spores of *T.thermophila* occurred in the form of intercalary or terminal swellings on the tips of lateral branches.

Recently, Latif *et al.* (1995) have studied the cellulolytic activity of *T.thermophila* and have found out that this fungus produced FPA, CMCase, β-glucosidase, xylanase and β-xylosidase, though the enzyme activities were moderate compared with other thermophilic species. Nevertheless, this study showed that *T.thermophila* might use cellulose as well as hemicellulose and also utilize raw lignocellulosic materials such as straw.

Xylanolytic activity and xylose utilization of *T.thermophila* was also studied. While the xylanolytic activities were moderate, *T.thermophila* was able to consume 90-93 % of the xylose provided in the medium (Banerjee *et al.*, 1995).



(a) (b)



(c)

Fig. 1.4 The intercalary (a, c), and terminal (b) swellings in the hyphae of *T.thermophila* (Tütek and Ceylan, 1996, Ögel *et al.*, 1998).

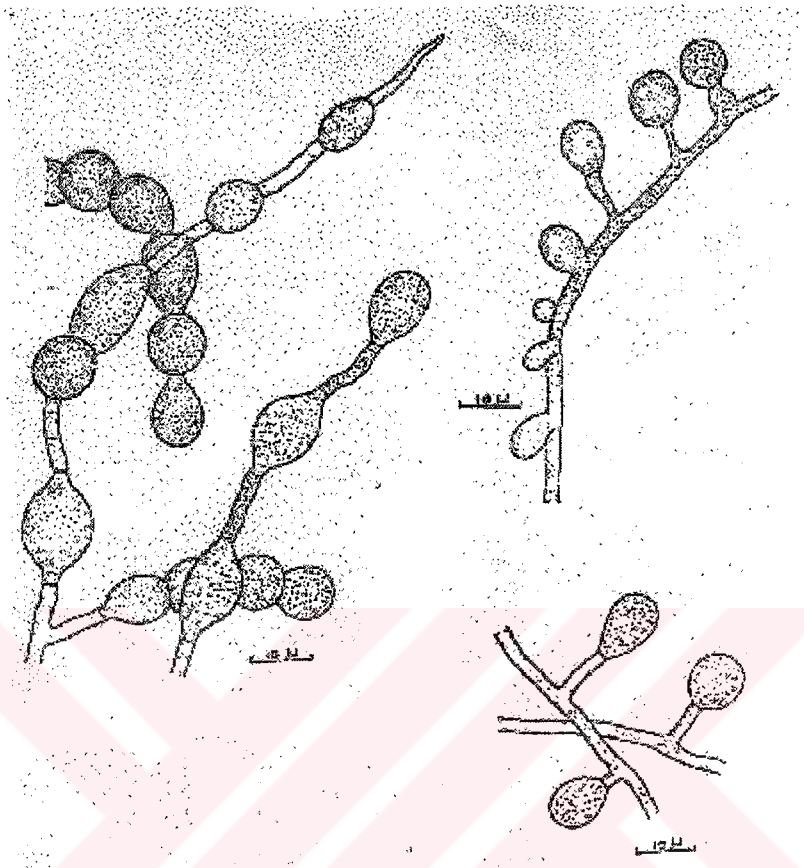


Fig. 1.5 Spore structures of *H.insolens* (Cooney and Emerson, 1964)

1.6 Effect of Medium Composition and Growth Conditions on the Production of Cellulases:

The growth and metabolism of microorganisms is greatly dependent on the environment in which they are found. Therefore, the specificity of the growth environment has to be taken into consideration when a certain organism is desired to be grown and made to produce some metabolites.

Detailed studies on the production of cellulases have revealed that growth of a specific microorganism and its enzyme production is greatly dependent on nutritional and environmental factors. Changes in the medium composition and growth conditions, including carbon source, nitrogen source, minerals and vitamins, oxygen supply, pH, temperature, and water content have been found to affect growth and cellulase productivity. Therefore, in order to find the optimum conditions for maximum cellulase production, these factors have to be investigated and thus, optimization of these parameters should be done.

1.6.1 Carbon Source

Fungi, in general, can utilize a variety of carbohydrates, including glucose, starch, cellulose and hemicellulose. While considering the production of cellulases, usually, cellulosic substrates are taken into account, because for some fungi it was found that cellulose had an inductive effect.

When considering the success of fermentation for cellulase production, the most significant factor is the nature and type of the carbon source (C-source) used. In laboratory scale, mostly pure celluloses such as Avicel, Solca Floc, cellulose powder, cotton or sulfite pulp are used. Usually, these substrates are good inducers, and good yields of cellulase are produced with them. But, because of the high cost of these substrates, they are not suitable for large-scale fermentations. Therefore, cheaper substrates are investigated for their effect on

cellulase productivity. These might be agricultural wastes such as wheat straw, wheat bran, maize stems, sorghum bagasse, sugar-beet pulp, and so on. In general, these lignocellulosic materials are found to produce lower cellulose yields when compared with pure celluloses, but there are also exceptions.

Kawamori *et al.* (1987) have studied the effect of different carbon sources on the cellulase production of the thermophilic fungus *Thermoascus aurantiacus* A-131 and mesophilic *T.reesei* QM 9414. While for *T.reesei* QM 9414, Avicel was the best C-source, for *T.aurantiacus* A-131 alkali-treated bagasse, Walseth's cellulose, and xylan yielded higher CMCase activities than Avicel.

In order to increase the cellulase productivity in fermentations with lignocellulosic substrates, pure cellulose might be added in low concentrations to effect the induction of enzyme synthesis. For example, for *Trichoderma* sp. M_7, the EG production was studied using Micricel (pure microcrystalline cellulose) alone or in combination with lignocellulosic materials such as wheat straw, wheat bran, and maize stems. The results indicated that the combined substrates were better for cellulase production than a single substrate (Atev *et al.*, 1987).

Since low-cost lignocellulosic materials together with cellulose contain lignin, hemicellulose and other compounds which were difficult to utilize by organisms, some pretreatments were applied in order to observe the effect on cellulase yields. It was found out that pretreatments of lignocellulosics such as reduction of particle size (i.e.: milling) or alkali (NaOH or NH_3) treatment can improve the cellulase productivity.

Two basidiomycetes cultures *Polyporus* BH_1 and BW_1 were studied for their cellulase activities on sugar cane bagasse. Milled bagasse with different particle sizes (mesh No. 28, 35, 48, 65, 80) was used as a C-source. The results showed that higher yields were obtained with 65 and 80 mesh when compared with other meshes, indicating that powdered and finely distributed bagasse could be much more easily metabolized than coarser particles (Nigam and Prabhu, 1991).

Studies were done on the effect of untreated wheat straw, steamed wheat straw, and NaOH-treated and steamed wheat straw on the cellulase production by *T.reesei* MCG-77. When NaOH-treated and steamed wheat straw was used as a C-source, the highest yields were obtained, followed by steamed wheat straw and the lowest yield was with untreated wheat straw (Doppelbauer, *et al.*, 1987).

1.6.2 Nitrogen Source

Another nutritional factor which is highly effective on the growth and metabolite production of microorganisms is the nitrogen source (N-source). The type and kind of N-source might be used for the regulation of the metabolic activities of fungi. Organic and inorganic N-sources may have different effects on fungi, namely some may grow well on organic nitrogen, whereas others may grow on inorganic nitrogen. Therefore, the N-source used in the fermentation for the production of cellulases vary from organism to organism.

Aspergillus niger NCIM 1207 was grown in a medium containing various N-sources including $(NH_4)_2SO_4$, $NH_4H_2PO_4$, $NaNO_3$, KNO_3, casamino acids, proteose peptone, yeast extract, corn steep liquor, urea and others. Among the tested inorganic N-sources, $(NH_4)_2SO_4$ and $NH_4H_2PO_4$ were the best for cellulase production; while from the organic N-sources, proteose peptone and corn steep liquor gave the highest cellulolytic activities. While comparing the effect of inorganic and organic N-sources, it was concluded that organic nitrogen was better for cellulase production from *A.niger* NCIM 1207 (Gokhale et al., 1991).

The production of cellulases by *Trichoderma* spp. S_1 was studied by using organic and inorganic N-sources such as KNO_3, $(NH_4)_2SO_4$, NH_4NO_3, peptone, and urea. The results indicated that the production of the cellulase enzyme complex was greatly enhanced by inorganic N-sources, the highest

activities being obtained with NH_4NO_3 (ammonium nitrate) (Ali and Akhand, 1992).

1.6.3 Minerals and Vitamins

For a proper growth, all microorganisms require elements such as phosphorous, sulphur, magnesium, potassium, iron, copper and so on. Besides these, vitamins are also essential for some organisms. Since metabolic activities and enzyme production of microorganisms are growth-associated, the presence or absence, and the concentration of these compounds also affect the production of cellulolytic enzymes.

Trichoderma spp. S_1 was grown on different inorganic salts such as NaCl, KCl, K_2HPO4, $CaCl_2.2H_2O$, and $MgSO_4.7H_2O$. The study showed that $MgSO_4$ markedly increased EG activity but was not effective on exoglucanase and β-glucosidase production (Ali and Akhand, 1992).

Copper sulphate was added at different concentrations (2, 3, and 5 mg/ml) to the fermentation medium of *Polyporus* BH_1 and BW_1. The results indicated that for both species, as copper concentration increased, exoglucanase and endoglucanase activities decreased whereas β-glucosidase production was enhanced. Besides this, the effect of vitamin addition was investigated. The tested vitamins were ascorbic acid, biotin, niacin and thiamin. It was found that for *Polyporus* BW_1, all vitamins stimulated EG synthesis, while for *Polyporus* BH_1, none of these vitamins affected the exo- and endo-glucanase production (Nigam and Prabhu, 1991).

1.6.4 Oxygen Supply

Fungi may be aerobic, facultatively anaerobic and obligate anaerobes. The presence or absence of oxygen in the culture medium greatly affects

microorganisms' growth and metabolism according to the requirements of the specific species. Studies on the influence of O_2 supply upon cellulase production have been performed indicating that cellulolytic activity is affected by O_2 presence, absence, and concentration in the medium.

Strains of *S.thermophile* were grown in stationary flasks and in flasks on a gyratory shaker. When grown in stationary flasks, mycelia developed only on the surface of the liquid and cellulose remained insolubilized until the 12^{th} day of fermentation; while in shaken flasks the fungi grew well throughout the medium and almost no cellulose remained after the 2^{nd} day of incubation. The results showed that adequate aeration was important for proper growth, and rapid and complete degradation of cellulose (Bhat and Maheshwari, 1987).

1.6.5 pH

The pH of the fermentation medium is another factor which influences microbial growth and thus, cellulase production. Studies showed that different microorganisms grow at a particular pH range, and the optimal growth and cellulase production for every cellulolytic organism are dependent on the specific organism (and its growth medium).

The effect of fermentation medium pH on the cellulase production of the aquatic hyphomycetes *Lunulospora curvula* and *Flagellospora penicillioides* was investigated. The pH was tested in the range of 3.0 to 8.0. The results showed that for both fungi the pH value of 5.0 favored the production of FPA and CMCase (Chandrashekar and Kaveriappa, 1991).

Research on the pH effect when a certain microorganism is grown on different C-sources has revealed that the optimum pH was also substrate dependent. When *T.reesei* QMY-1 was grown on pure cellulose (alpha cellulose) pH of 5.0 was optimal for cellulase synthesis. But when the substrate was crude cellulose (chemithermomechanical pulp, CTMP), pH of 6.0 was optimal for FPA production. It was suggested that higher pH was favorable for

cellulase production on CTMP than on pure cellulose, because of the different chemical composition, since CTMP was composed of 60 % cellulose and the remainder was hemicellulose and lignin, while alpha cellulose was 99.9 % cellulose (Chahal *et al.*, 1992).

1.6.6 Temperature

Microorganisms grow at various temperature ranges, and according to their growth temperature ranges they are classified into psychrophilic, mesophilic, thermophilic, psychrotolerant and thermotolerant. The temperature at which a specific organism is grown affects growth parameters such as lag time, specific growth rate and total yield of metabolites. Therefore, to find the optimal temperature for growth, the effect of the range of temperatures between the maximum and minimum for growth on the parameters mentioned above has to be investigated.

In general, the specific growth rate is low near the minimum growth temperature and increases with an increase in temperature, until an optimum value is reached at which growth rate is maximum. Above the optimum temperature, the specific growth rate rapidly decreases and the maximum growth temperature is a few degrees above the optimum. The reasons for this might be denaturation of one or more key enzymes and/or breakdown of metabolic regulatory mechanisms.

Fungal growth at different temperatures might be expressed by using the Arrhenius equation:

$K = A.e^{-E/RT}$,

where R is the gas constant, T the absolute temperature, E the energy of activation, and A a constant dependent on the specific organism (Carlile and Watkinson, 1995).

Strains of *Sporotrichum thermophile* were grown at different temperatures in order to see the effect of temperature on cellulase production.

The results showed that some cellulose remained insolubilized in the flasks at 30°C, while in the flasks at 50°C virtually no cellulose was left. Sporulation was increased at 30°C. The FPA in the cultures grown at 30°C was in the range 0.08 – 0.10 U/ml, while in those at 50°C ranged from 0.10 to 0.13 U/ml (Bhat and Maheshwari, 1987).

The effect of growth temperature on CMCase production of *Thermoascus aurantiacus* A-131 was studied in L-shaped tubes and was compared with that of *T.reesei* QM 9414. *T.aurantiacus* A-131 produced CMCase in the range of 37–57°C and the maximum CMCase productivity was at 45-50°C. For *T.reesei* QM 9414, the maximum productivity of CMCase was in the range of 23-30°C (Kawamori *et al.*, 1987).

1.6.7 Water Content

Water content of the fermentation medium of any microorganism greatly influences its growth and metabolism, since organisms are composed largely of water. To express the availability of water in the medium, generally, a term known as water activity (a_w) is used. Water activity is the ratio between the water vapor pressure of the solution being considered (p_s) and that of pure water (p_w), ranging between 0.0 and 1.0, and is expressed by the following equation:

$a_w = p_s/p_w$ (Carlile and Watkinson, 1995).

According to the water content, fermentations might be divided into submerged, semi solid-state and solid-state cultivations. Usually, microorganisms grow differently in these media and thus, their metabolic activities are different. Studies on the cellulase production in submerged and solid-state fermentations have shown that in both media, cellulases are produced, the enzyme activities being different.

Dueñas *et al.* (1995) have grown a mixed culture of *Trichoderma reesei* LM-UC4 and *Aspergillus phoenicis* QM 329 on ammonia-treated bagasse with 80 % (w/w) moisture. It was suggested that though solid-state fermentation was a technology with lower overall productivity and difficult process control, it would be a suitable process for industrial cellulase production with agricultural wastes especially for the developing countries, since the technology was simple and with low capital cost for equipment.

The cellulase synthesis by *Trichoderma* sp. M_7 was studied in submerged and solid-state cultivation conditions. In both fermentation types, cellulases were produced. When the volumetric amount of cellulases was considered, submerged cultivation gave higher values, but the exocell protein was higher in solid-state fermentation (Atev *et al.*, 1987).

1.7 Effect of pH and Temperature on the Cellulase Enzyme Activities

Studies have shown that pH and temperature greatly affect the activity of any enzyme. The characteristics of the structural components of enzymes, i.e. amino acids, depend on pH and thus, pH changes affect enzymatic activities. Generally, the active sites of enzymes consist of ionizable groups which have to be in the proper ionic form in order to achieve the desired catalysis. At very low or high pH values, the tertiary structure of the enzymes may be disrupted and the proteins denatured. Also, the degree of ionization of some amino acid chains may depend on the medium pH and/or the substrate may contain ionizable groups only one of which can be hydrolyzed by the specific enzyme. Therefore, to achieve the desired enzymatic reaction at high rate and productivity, the effect of pH of the reaction medium must be studied to find the optimum value (Palmer, 1991).

For enzymatic reactions, it was observed that with increase in temperature the reaction rates also increase up to a certain value after which a

sudden decrease occurs. The catalytic activity of any enzyme (as mentioned above) is dependent on the configuration of the ionizable groups in the active site constituting the tertiary structure of the enzyme. As temperature increases, more collisions occur per unit time and the ionizable groups are set in the proper configuration. At a certain temperature (dependent on the specific enzyme from a specific organism) where the enzyme activity is maximum, this temperature is termed as the optimum temperature for enzymatic activity. After this, usually, there is a sudden decrease in the enzyme activity due to the disruption of the tertiary structure, and further denaturation of the enzyme.

Studies on the effect of pH and temperature on the activities of cellulolytic enzymes have shown that they are also pH and temperature dependent. The effect of pH and temperature on the cellulases of *Thermoascus aurantiacus* A-131 was determined in the range of pH 2.5–9.0 and 30–80°C. The optimum temperature for Avicelase, CMCase and β-glucosidase were 70°C, 70°C and 75°C, respectively; while the optimum pH values were 4.5 for Avicelase and CMCase, and 5.0 for β-glucosidase (Kawamori *et al.*, 1987).

The cellulolytic enzyme activities produced in the culture filtrates of *Lunulospora curvula* and *Flagellospora penicillioides* were tested at different temperatures (16–40°C) and pH values (3.0–8.0). For both fungi, the optimum temperature and pH for FPA and CMCase activities were 35°C and 5.2, respectively (Chandrashekar and Kaveriappa, 1991).

Exoglucanase, endoglucanase and β-glucosidase of *Trichoderma* spp. S_1 were partially purified from the culture supernatant. The effect of pH on the activity of these enzymes was determined in the range of 2.0 to 7.0. EG and exoglucanase were optimally active at pH 3–4, while β-glucosidase – at pH 5–6. The analysis of the effect of temperature on enzyme activity (40–80°C) revealed that β-glucosidase and exoglucanase had maximum activities at 50°C, while EG showed a peak at 60°C (Ali and Akhand, 1992).

The effect of temperature and pH on the cellulases of *Trichoderma* sp. A-001 was investigated at 40–80°C and pH 4.0–8.0. CMCase, FPA and β-

glucosidase of the culture supernatant exhibited maximum activities at pH 5.5 and 60°C (Gashe, 1992).

CMCase from *Trichoderma viride* was purified and characterized. The enzyme activity was dependent on various factors such as enzyme and substrate concentration, incubation time, pH and temperature. The optimum temperature for maximal CMCase activity was 45°C, and the optimum pH was 5.0 (Olama *et al.*, 1993).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Torula thermophila strain 3A was isolated from mushroom compost (Tütek and Ceylan, 1996, Ögel *et al.*, 1998). The industrial strain of *Humicola insolens*, as well as commercial cellulase from *H.insolens* of Novo Nordisk and cellulase of ORBA were obtained from ORBA Inc.. Avicel (No: 2331, microcrystalline cellulose for column chromatography) (Merck), CMC (carboxymethyl cellulose) (Sigma) and all other chemicals were of analytical grade.

2.2 Methods

2.2.1 Maintenance and Cultivation of Strains

Stock cultures of *H.insolens* and *T.thermophila* were grown on YpSs agar slants at 37°C, and maintained at room temperature. Stock cultures were subcultured every two months. The composition of the YpSs agar was: Difco powdered yeast extract, 4.0 g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; soluble starch, 15.0 g; agar, 20.0 g in 1 L distilled water (Cooney and Emerson, 1964).

Shake-flask culture experiments were carried out in 2 L Erlenmeyer flasks with 1L of fermentation medium, containing modified YpSs medium with microcrystalline cellulose (Avicel) as the carbon source, instead of starch (Avicel, 20.0 g; Difco powdered yeast extract, 4.0 g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g in 1 L distilled water), or modified Czapek's medium (Avicel, 20.0 g; $NaNO_3$, 3.0 g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; KCl, 0.5 g in 1 L distilled water) (Cooney and Emerson, 1964). The pH of the fermentation medium was adjusted to 7.5 with 0.1 M H_3PO_4 or 0.1 M KOH. To compare the cellulolytic activity of the newly isolated strain of *T.thermophila* with that of the industrial strain of *H.insolens*, all the experiments were run in parallel with both microorganisms, and by starting with the same amount of spores. 3 mg of spores suspended in sterile distilled water were used to inoculate 20 ml of preculture medium in 50 ml Erlenmeyer flasks, containing YpSs with 1 % glucose as the carbon source, instead of starch. Precultures were incubated for 24 hr at the stated main culture growth temperature. After 24 hr of incubation these precultures were used to inoculate 1 L main cultures, prepared as described before. The experiments were carried out in a shaker incubator at 155 rpm and the growth temperatures ranged from 35°C to 55°C (with 5°C±1°C increments) in order to determine the optimum temperature for enzyme production. The protein determination and the enzyme activity measurements were carried out every 24 hr starting from the second till the eighth day of incubation, while substrate consumption (in terms of the amount of insoluble cellulose in the culture medium) was determined starting from the first till the eighth day of incubation.

For the enzyme activity, protein content and growth measurements, samples were removed every 24 hours and centrifuged at 3000 rpm for 20 min. After centrifugation, the supernatant was used for the measurements of enzyme activities and total exocell protein content, while the pellet was used for the determination of fungal biomass generation and substrate consumption.

2.2.2 Cellulase Activity Measurements

2.2.2.1 Activity Measurements in Culture Supernatants

In order to determine cellulase activities, methods based on the measurement of releasing sugar content were used. In the experiments, total cellulase, endoglucanase and Avicel-adsorbable endoglucanase activities were determined.

Total cellulase activity was measured by using filter paper as the substrate. Strings of filter paper (Whatman No: 1, 1×6 cm) were ruled as small cylinders and put in the test-tubes containing 0.5 ml of 25 mM sodium acetate buffer (at pH=6.0 and 6.5, for *T.thermophila* and *H.insolens*, respectively). Further, these test-tubes (covered with lids) were placed in a water bath at 60°C for *H.insolens* and 65°C for *T.thermophila.* The temperature and pH used in enzyme assays were experimentally determined as optimal (See Sec. 3.2 and 3.3). When the mixtures in the test-tubes were heated to the desired temperature, 0.5 ml of appropriately diluted supernatant (enzyme solution) was added to each of the tubes. Dilutions ranged from 1/2 to 1/30. The tubes were incubated at the stated temperature for 30 min. Then, to stop the reaction, 2 ml of a mixture of Somogyi I and Somogyi II reagents (Appendix B) were added. Aliquots were removed into dry tubes, immediately placed into boiling water bath and kept for 15 min. The tubes were then cooled to room temperature. Further, 2 ml of Nelson reagent (Appendix B) was added into each tube and the contents were immediately mixed on a Vortex mixer. Following mixing, 4 ml of distilled water was added into the tubes and the suspensions were mixed. The contents of the tubes were transferred into 15 ml Falcon tubes, and centrifuged at 5000 rpm for 10 min to remove any remaining insolubles. Optical densities were measured at 520 nm against a reagent blank prepared by using buffer instead of the culture supernatant, with the remaining of the procedure being the same (Wood and Bhat, 1988). The absorbance values were

used to calculate the enzyme activities by using a glucose standard curve (Appendix A). The experiments were carried in duplicate and the results were taken as the mean of the two data. The enzyme activity per ml supernatant was measured by using the following equation:

$$EU/ml = (X.D)/(M.t),$$

where X is the amount of reducing sugar in terms of μg of glucose, D the dilution factor, M the molecular weight of glucose (180 g/mol) and t the time of incubation at assay conditions (30 min.). Therefore, one unit of total cellulase activity was defined as the amount of enzyme required to release 1 μmol glucose equivalents per minute under the specified assay conditions.

Specific activities (EU/mg protein) were calculated by dividing the enzyme activity (EU/ml) to the amount of total protein in 1 ml. Likewise, enzyme activity per g biomass was determined by dividing the enzyme activity (EU/ml) to the weight of biomass in 1 ml.

Endoglucanase activity was measured by using carboxymethyl cellulose (CMC) as the substrate. Solution containing 1 % (w/v) CMC was prepared. 1 ml of substrate solution and 0.5 ml of 25 mM sodium acetate buffer, pH 6.0 for *T.thermophila* and pH 6.5 for *H.insolens* were mixed in the test tubes and heated to the desired temperature (60°C for *H.insolens* and 65°C for *T.thermophila*). Further, 0.5 ml of appropriately diluted supernatant (enzyme solution) was added. After incubation at the stated temperatures for both of the microorganisms for 30 min., the enzymatic reaction was stopped by the addition of 2 ml of a mixture of Somogyi I and Somogyi II reagents (given in Appendix B). Then, the reducing sugar content was measured and the enzyme activity was calculated as explained above (Wood and Bhat, 1988). One unit of endoglucanase activity (per ml of the culture supernatants) was defined as the amount of enzyme required to release 1 μmol glucose equivalents per minute (per ml of the culture supernatant) under the stated assay conditions.

2.2.2.2 Activity Measurement in Commercial Preparations

250 mg of dry sample (Novo Nordisk or ORBA cellulase) were mixed with 50 ml of distilled H_2O. After stirring for 15 min., the preparation was left for 15 min. at room temperature. Further, samples from the preparation were taken and the endoglucanase activity and total protein were determined as explained in Sections 2.2.2.1 and 2.2.6, respectively. (For the endoglucanase activity measurements the assay conditions were pH 6.5 and 60°C.)

2.2.3 Adsorption of Endoglucanase onto Avicel

5 ml of 25 mM sodium acetate buffer (pH 6.0 for *T.thermophila* and 6.5 for *H.insolens*) and 1.0 g Avicel were mixed in a 50 ml Falcon tube and cooled to 4°C. Previously cooled 5 ml of culture supernatant was added to the prepared solution, mixed and kept at 4°C for 15 min., the tubes being placed horizontally (Ali *et al.*, 1993). After centrifugation, the endoglucanase activity in the supernatant was measured (as explained in Sec. 2.2.2). Avicel-adsorbable endoglucanase was measured indirectly by subtracting the activity of Avicel-nonadsorbable endoglucanase from the activity of total endoglucanase. Again, one unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of glucose equivalents per minute per ml of the culture supernatant under the assay conditions.

2.2.4 Effect of Assay pH

The pH of the buffer used in the assay for measuring the endoglucanase activity was varied from 4.5 to 9.0, with 0.5 increments. From 4.5 to 6.5, 25mM sodium acetate buffer, while from 7.0 to 9.0, 25mM sodium phosphate buffer was used and the assay temperature was kept constant at 55°C.

2.2.5 Effect of Assay Temperature

The endoglucanase activity was assayed at different temperatures in the range of 35 to 75°C, with 5°C increments, and with a constant assay pH of 6.0 for *T.thermophila* and 6.5 for *H.insolens.*

Percent relative activity was determined in the experiments for the effects of assay pH and temperature by taking the maximum value as 100 % and all other values were calculated as the percentage of the maximum activity of the given set of experimental data.

2.2.6 Protein Measurement

The total exocell (extracellular) protein found in the supernatant was measured by Lowry's method (Lowry, 1951). 75 µl of appropriately diluted supernatant was placed into Eppendorff tubes. 750 µl of reagent C (Appendix D) was added and the solution was mixed. The mixture was left for 20 min. at room temperature. 75 µl of Folin phenol reagent D (Appendix D) was added and the solution was mixed. The mixture was left for 1 hr at room temperature and the optical density was measured at 750 nm against a reagent blank

prepared by using buffer solution instead of the supernatant and all other treatments were applied. The absorbance values read from the spectrophotometer were used to calculate the protein content by using a BSA (bovine serum albumin) standard curve (Appendix C). All the experiments were carried in duplicate and the results were taken as the mean of the two data. Exocell protein was expressed as mg BSA per ml culture supernatant.

2.2.7 Determination of Substrate Consumption and Biomass Generation

The determination of fungal biomass generation is essential for the proper interpretation of data and for kinetic modelling studies. Fungal biomass can be determined by subtracting the amount of insoluble cellulose in the culture filtrate from the weight of the total biomass (the culture filtrate).

The cellulose present in the dry pellet was analyzed by using the method of Updegraff for the determination of cellulose in biological materials (Updegraff, 1969). 20 ml samples were taken from the culture, centrifuged at 3000 rpm for 20 min, and the resulting pellet was dried at 100°C for 3–4 hr and then overnight at 70°C. The dry pellet was weighed and placed into 50 ml Falcon tubes. 3 ml of acetic-nitric reagent (Appendix F) was added. This was done by adding 1 ml of the reagent, mixing well on a Vortex mixer, and adding the remaining 2 ml and remixing. The tubes with the mixtures were placed in a boiling water bath for 30 min. Then, the tubes were centrifuged at 5000 rpm for 7 min., and the supernatant was discarded. 10 ml of distilled water was added, by adding 3 ml of distilled water, mixing well on a Vortex mixer, then adding the remaining 7 ml and remixing again. After centrifugation at 5000 rpm for 7 min., the supernatant was discarded and 10 ml of 67 % H_2SO_4 (v/v) was added, by first adding 3 ml of H_2SO_4, mixing well on a Vortex mixer, then adding the remaining 7 ml and mixing. The mixture was left for 1 hr at room temperature and diluted with distilled water (dilution range (1/100 to 1/200). 1 ml of this

dilution was mixed with 4 ml of distilled water in a test-tube. After cooling the tubes in an ice bath, 10 ml of cold anthrone reagent (Appendix F) was added by layering with a pipette. The solution was mixed well on a Vortex mixer and the tubes were kept in the ice bath until all the tubes were mixed. Further, the tubes were placed in a boiling water bath for 16 min. After that, the tubes were cooled in ice bath for 3 min. and then left at room temperature for 10 min. The optical density was measured at 620 nm against a reagent blank by using distilled water instead of cellulosic solution and starting the treatments after the dilution of the samples. The absorbance values read from the spectrophotometer were used to calculate the cellulose content by using a cellulose standard curve (Appendix E). All the experiments were carried out in duplicate and the results were taken as the mean of the two data. The cellulose concentration per ml of culture medium was determined by using the dilution factors.

The fungal biomass was determined indirectly by subtracting the amount of insoluble cellulose from the amount of total biomass, which corresponds to the weight of the culture filtrate obtained from 1 ml of culture medium:

Fungal Biomass (mg/ml)=Total Biomass (mg/ml) – Cellulose content (mg/ml)

CHAPTER 3

RESULTS AND DISCUSSION

The aim of this study was to analyze and compare the cellulase activity and growth of the newly isolated strain of *T.thermophila* and the industrial strain of *H.insolens*. Two different cultures were used and the parameters considered were assay pH, assay temperature and growth temperature. Total cellulase, endoglucanase (EG) and Avicel-adsorbable endoglucanase (AAEG, endoglucanase which can be adsorbed onto Avicel) activities together with exocell protein, biomass generation and medium pH have been measured with respect to time during the cultivation of the microorganisms.

3.1 Selection of a Medium for Cellulase Production

The first step of the study was the selection of an appropriate culture medium for cellulase production by *T.thermophila* and *H.insolens*. Cooney and Emerson (1964) have suggested several culture media which have proved to be suitable for growing especially thermophilic fungi. These were: Yeast-starch (YpSs) agar, Yeast-glucose (YG) agar, Oatmeal agar, and Czapek's agar. From these, YpSs and Czapek's were selected for use in cultivations. The contents of these culture media have been modified by excluding the agar, and using 2 % Avicel (microcrystalline cellulose) as the C-source instead of glucose

(Czapek's) or starch (YpSs). The compositions of the modified YpSs and Czapek's media are given in Sec. 2.2.1.

Both *T.thermophila* and *H.insolens* were grown on 250 ml of YpSs and Czapek's media at 40°C, the EG and FPA activities were measured. Growth temperature of 40°C was selected since in previous studies it has been found that *T.thermophila* and *H.insolens* grow most rapidly at about 40°C (Cooney and Emerson, 1964). In the measurement of cellulase activities, assays were carried out at pH 6.0 and 55°C. These assay conditions were provisionally selected, as they have proved to be suitable for the determination of cellulase activities of *H.insolens* (M.Batum, personal communication). The maximum FPA and EG activities obtained upon growth of *T.thermophila* and *H.insolens* on modified YpSs and Czapek's media are given in Table 3.1.

From Table 3.1, it can be seen that both the FPA and EG activities of *T.thermophila* are much higher in the modified YpSs than in the modified Czapek's medium. EG activity in the modified YpSs was *c.* 27 times higher than that in the modified Czapek's medium, and FPA was *c.* 14 times higher in the modified YpSs. Therefore, it was concluded that the modified YpSs medium was better for the production of cellulases by *T.thermophila*.

The results in Table 3.1 also show that for *H.insolens*, the EG and FPA in the modified YpSs were *c.* 8 times and *c.* 26 times higher, respectively than those obtained upon growth in the modified Czapek's medium. Thus, for *H.insolens*, again the modified YpSs medium was found to be better for the production of cellulases.

Higher cellulase activities were obtained when the organisms were grown on the modified YpSs medium in terms of U/ml; but since the fungal biomass was not estimated in this set of experiments, the effect of fermentation medium on the growth of the fungi cannot be revealed.

When the contents of the YpSs and Czapek's media (Sec. 2.2.1) are analyzed, it is seen that the main difference is the type of the N-source and the presence of KCl and $FeSO_4$ in the modified Czapek's medium. While in the

Table 3.1 Production of cellulases by *T.thermophila* and *H.insolens* in different media

	T.thermophila		*H.insolens*	
	EG (U/ml)	FPA (U/ml)	EG (U/ml)	FPA (U/ml)
Modified YpSs Medium	1.407	0.069	1.389	0.129
Modified Czapek's Medium	0.052	0.005	0.174	0.005

Czapek's medium the N-source is inorganic ($NaNO_3$), that of the YpSs medium is organic (yeast extract). The results showed that the cellulase production of *T.thermophila* and *H.insolens* was favored by organic N-source. Besides offering N-source, yeast extract may also contain some trace amounts of other essential nutrients such as vitamins. Similar results have been obtained by Ali *et al.* (1993), when *Humicola* spp. H_1 was cultivated on different N-sources. It was suggested that yeast extract had a stimulatory effect on the synthesis of cellulases in *Humicola* spp. H_1.

When the enzyme activities of *H.insolens* and *T.thermophila* in the YpSs medium (Table 3.1) are compared, it can be seen that *H.insolens* produced higher FPA (*c.* 2 times higher) than *T.thermophila*, while the EG activities were similar, that of *T.thermophila* being slightly higher. The results indicated that the newly isolated *T.thermophila* was as good EG producer as the industrial strain of *H.insolens* under the stated conditions.

In concern with the results obtained in these experiments, in the following steps of the study the modified YpSs medium was used as a fermentation medium since in this medium both fungi produced higher cellulase activities.

3.2. Effect of Assay pH on the Endoglucanase Activity of *T.thermophila* and *H.insolens*

The effect of the assay pH on the activity of EG of *T.thermophila* and *H.insolens* was analyzed. The pH range in which the EG activity was measured was 4.5–9.0. The enzyme activity was measured at 55°C and time of incubation for the enzymatic reaction was kept constant as 30 min. As it is well known, the activity of any enzyme is greatly affected by pH, thereby it is quite important to learn at which pH the specific enzyme exhibits maximum activity. For this purpose, *T.thermophila* and *H.insolens* were grown at 40°C and 155 rpm and the EG activities were determined. The plots given in Fig. 3.1 and Fig. 3.2 indicate that for *T.thermophila* the optimum assay pH for EG activity is at pH 6.0, while that of *H.insolens* is at pH 6.5.

For *H.insolens*, in the 4th day of incubation, the maximum EG activity was observed at pH 6.0, but in the remaining three days the maximum activity was at pH 6.5 (Fig. 3.2). The differences between the % relative activities during the different days of incubation might be due to the synthesis of different EGs during the cultivation. Schülein (1997) has characterized five different EGs from a strain of *H.insolens* and their pH profiles indicated that these enzymes show maximum activity at different pH values. It is likely that *T.thermophila* also secretes a number of different EGs as this organism is taxonomically closely related to *H.insolens* (Cooney and Emerson, 1964). Although there is no information, at present, in the literature to support this suggestion, it is clear from the results in section 3.4 that the EGs secreted by *T.thermophila* are composed of at least two types, namely, Avicel-adsorbable and Avicel-non-adsorbable EGs.

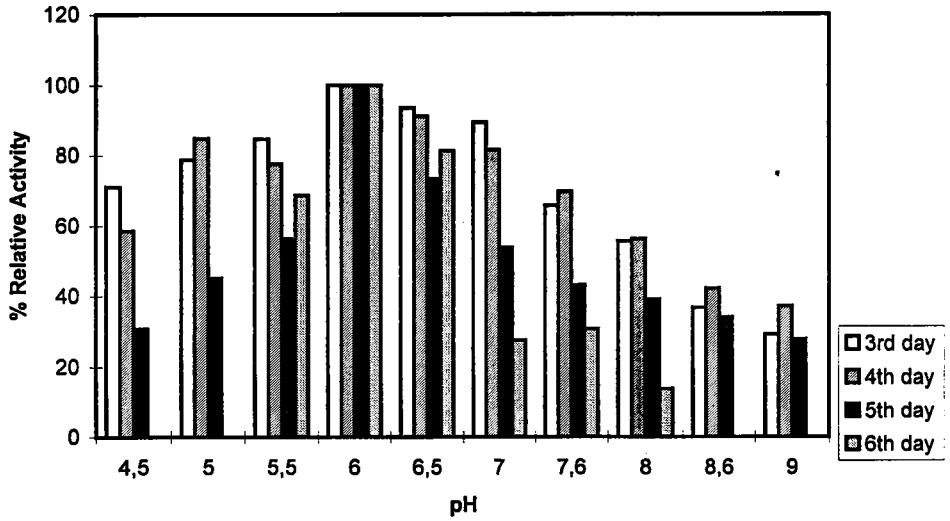


Fig. 3.1 Effect of assay pH on the EG activity of *T.thermophila*

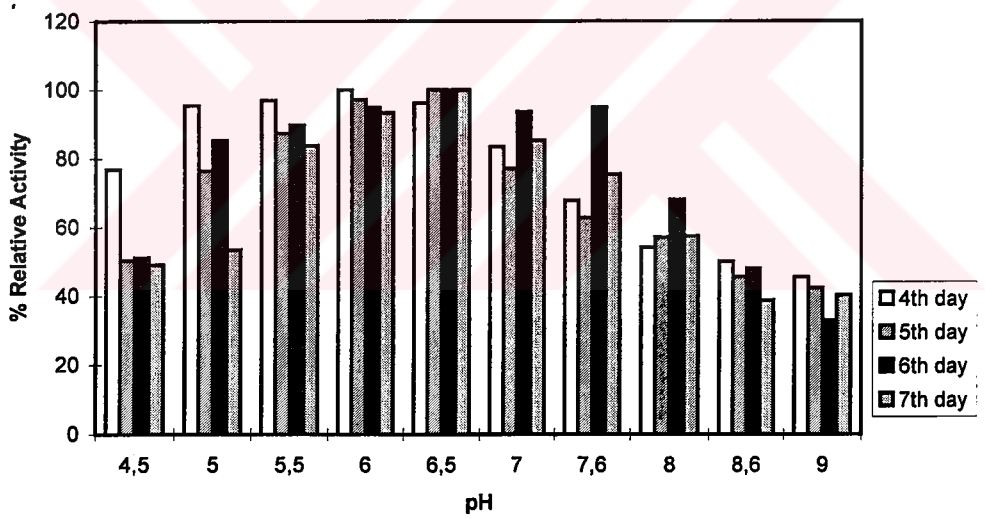


Fig 3.2 Effect of assay pH on the EG activity of *H.insolens*

When considering the results in Fig. 3.1 and 3.2, it can be concluded that the pH optima of EGs of *T.thermophila* and *H.insolens* are 6.0 and 6.5, respectively, being near neutrality. The pH profile of the EG of *H.insolens* was flatter when compared with that of *T.thermophila*, since *H.insolens* retained around 80 % of its activity in the pH range 5.5 – 7.0, while this is not the case for *T.thermophila* EG.

In industrial applications, the optimum pH of an enzyme carries a great significance since especially in food industry various products have a certain pH which might affect the enzymatic activities. Therefore, the pH of a given enzyme indicates its range of applications, since for various processes different pH values are desired. For example, for extraction of fruit juices, the pH depends on the specific raw material (fruit) being used.

In studies on fungal cellulases, the optimum pH for EGs were found to be 4.0 for *Trichoderma* spp. S_1 (Ali *et al.*, 1992), 5.0 for *Trichoderma viride* (Sharma *et al.*, 1991), 5.5 for *Trichoderma* sp. A-001 (Gashe, 1992) 5.0 for *H.grisea* var. *thermoidea* (Hayashida *et al.*, 1988), 4.5 for *Thermoascus aurantiacus* A-131 and *Trichoderma reesei* QM 9414 (Kawamori *et al.*, 1987). When comparing these results with those obtained for the EGs of *T.thermophila* and *H.insolens*, it can be concluded that, in both cases, the pH optima are more close to neutrality. Depending on the pH desired in specific applications, the EGs of *T.thermophila* and *H.insolens* might be used where their optimum pH values are suitable.

3.3 Effect of Assay Temperature on the Endoglucanase Activity of *T.thermophila* and *H.insolens*

In order to find out the effect of assay temperature on the activities of EGs of *T.thermophila* and *H.insolens*, EG activities were determined for the

temperature range of 30–75°C, while the time of incubation for the enzymatic reaction was 30 min. Accordingly, the optimum temperature at which *T.thermophila* EG showed maximum activity was 65°C (Fig. 3.3), while for *H.insolens* EG the temperature optimum was found as 60°C (Fig. 3.4).

In studies on the EGs of other organisms, the optimum temperatures for EG activity were found to be 50°C for *Trichoderma reesei* QM 9414, 50°C for *H.grisea* var. *thermoidea* (Hayashida *et al.*, 1988), 70° for *Thermoascus aurantiacus* A-131 (Kawamori *et al.*, 1987), and 45°C for *Trichoderma viride* (Olama *et al.*, 1993).

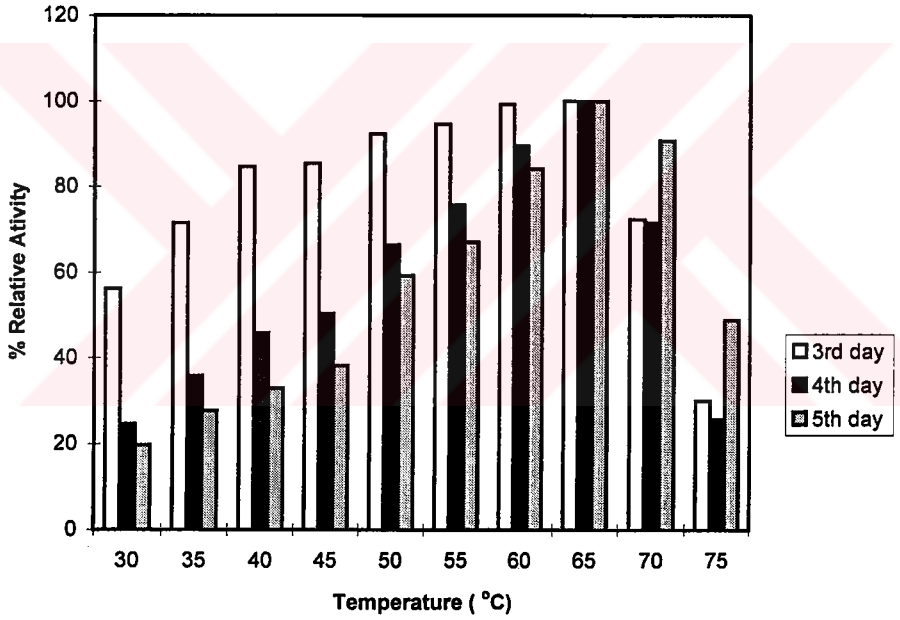


Fig 3.3 Effect of assay temperature on the EG activity of *T.thermophila*

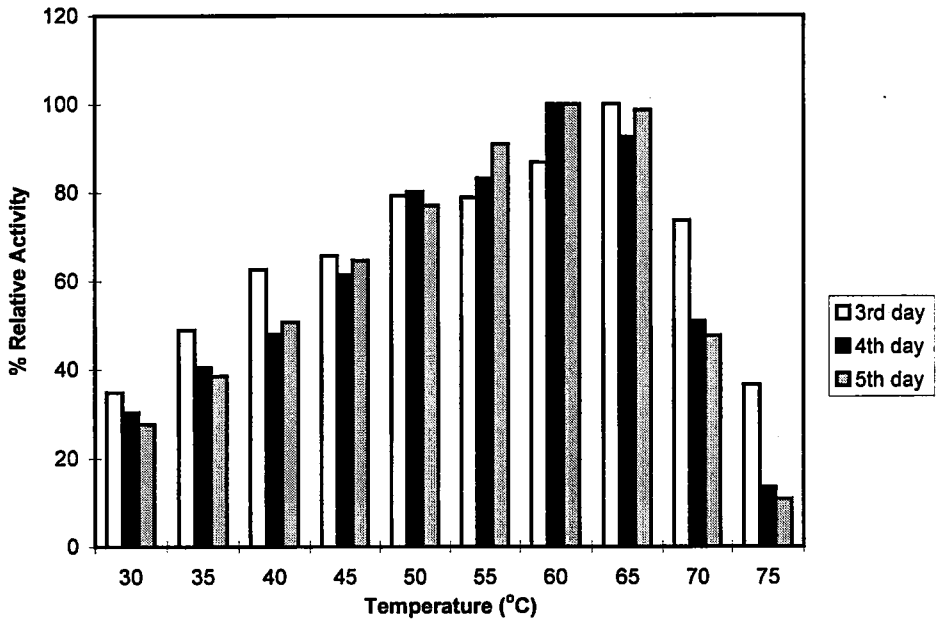


Fig. 3.4 Effect of assay temperature on the EG activity of *H.insolens*

When these results are compared with those of *T.thermophila* and *H.insolens*, it is obvious that the temperature optima for *T.thermophila* and *H.insolens* EGs are higher than those of *Trichoderma* and *H.grisea* var. *thermoidea* EGs, but less than those of *T.aurantiacus*.

In certain industrial applications, higher temperatures are desired in order to reduce contamination and achieve aseptic conditions. In processes such as animal feed production, waste treatment, pulp and paper manufacture, thermostable enzymes are required. In such treatments with thermostable enzymes, the higher the temperature, the safer the process will be.

3.4 Effect of Growth Temperature on the Cellulase Production and Growth of *T.thermophila* and *H.insolens*

In order to establish the effect of growth temperature on cellulase, and specifically endoglucanase by *T.thermophila* and *H.insolens* the FPA, EG and AAEG activities as well as fungal biomass, exocell protein and changes in medium pH were determined at different growth temperatures ranging from 35 to 55°C. Primarily the EG activity was considered, since with the developments in food technology this enzyme gains significance in applications such as extraction and clarification of fruit juices. Furthermore, EGs find use in textile industry in obtaining worn-outlook of jeans. The exoglucanase and β-glucosidase activities were not measured.

3.4.1 Effect of Growth Temperature on the Cellulase Production by *T.thermophila* and *H.insolens*

The production of FPA, EG and AAEG by *T.thermophila* with respect to cultivation time and growth temperature are given in figures 3.5, 3.6 and 3.7, respectively. The results indicate that cellulolytic enzymes are produced by *T.thermophila* in the range of 35–55°C, and 40–50°C appears to favor cellulase production. The maximum FPA and EG production was at 45°C, and maximum AAEG was at 50°C, although the values at 45 and 50°C were nearly identical. Therefore, it could be concluded that maximum EG and AAEG are produced in the range of 45–50°C under the specified cultivation conditions.

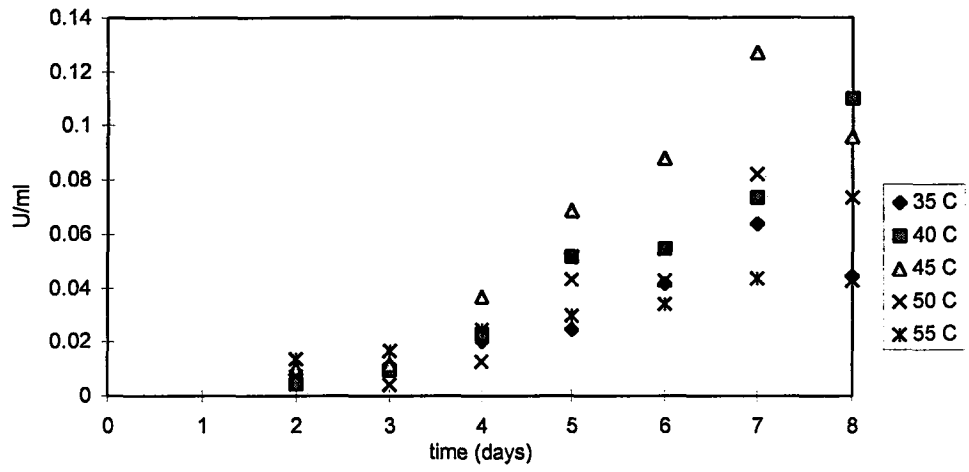


Fig 3.5 Time course of FPA production by *T.thermophila* at different growth temperatures.

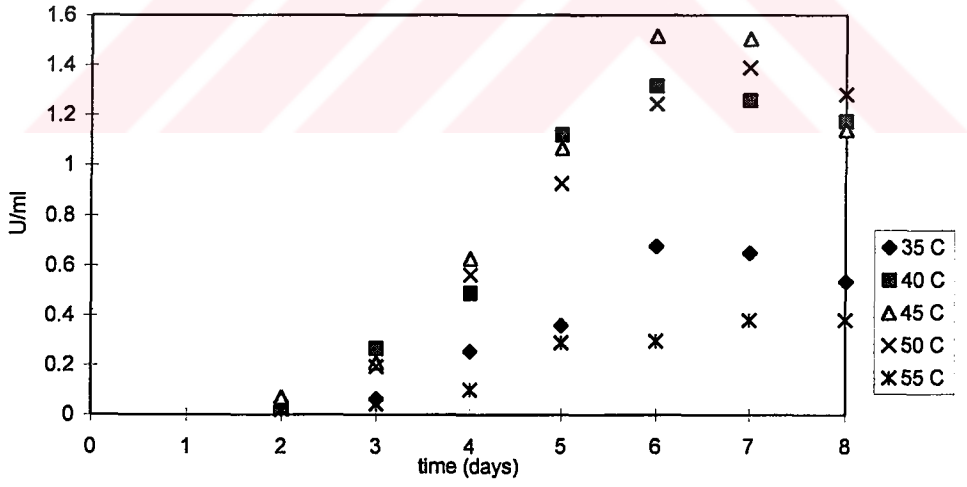


Fig. 3.6 Time course of EG production by *T.thermophila* at different growth temperatures

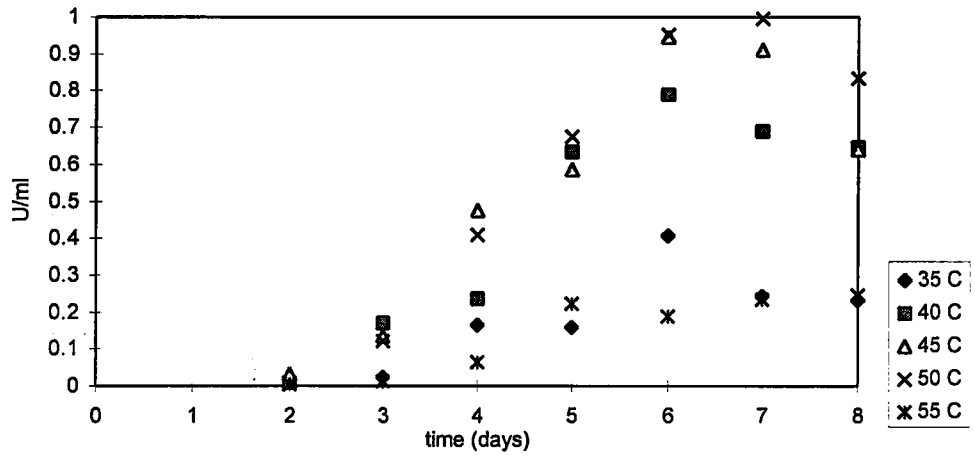


Fig 3.7 Time course of AAEG production by *T thermophila* at different growth temperatures.

The production of FPA, EG and AAEG by *H.insolens* at different growth temperatures are given in figures 3.8, 3.9 and 3.10, respectively. *H.insolens* also produced cellulolytic enzymes in the range of 35–55°C, and again 40–50°C seemed to be optimal for cellulase production. The maximum FPA and AAEG was obtained at 50°C, although activities of AAEG were found to be similar within the range of 40–50°C (Fig.3.10). Maximum levels of EG were produced at 45°C, however activities were almost the same at 45 and 50°C (Fig. 3.9). FPA of *H.insolens* was markedly higher at 50°C (Fig. 3.8).

Kawamori *et al.* (1987) have studied the effect of cultivation temperature on the production of CMCase by *T.aurantiacus* A-131. The results have revealed that this thermophilic fungus produced CMCase in the range of 37-57°C and produced maximum CMCase in the range of 45-50°C. These results are in consistence with those obtained for *T.thermophila* and *H.insolens.*

The fact that changes in the growth temperature from 40 to 50°C did not have a significant effect on enzyme production might be an advantage in the

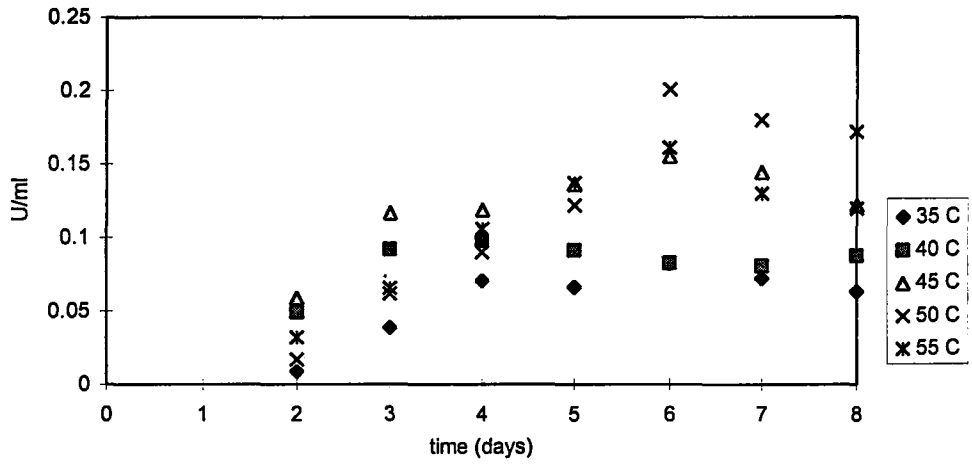


Fig. 3.8 Time course of FPA production by *H.insolens* at different growth temperatures.

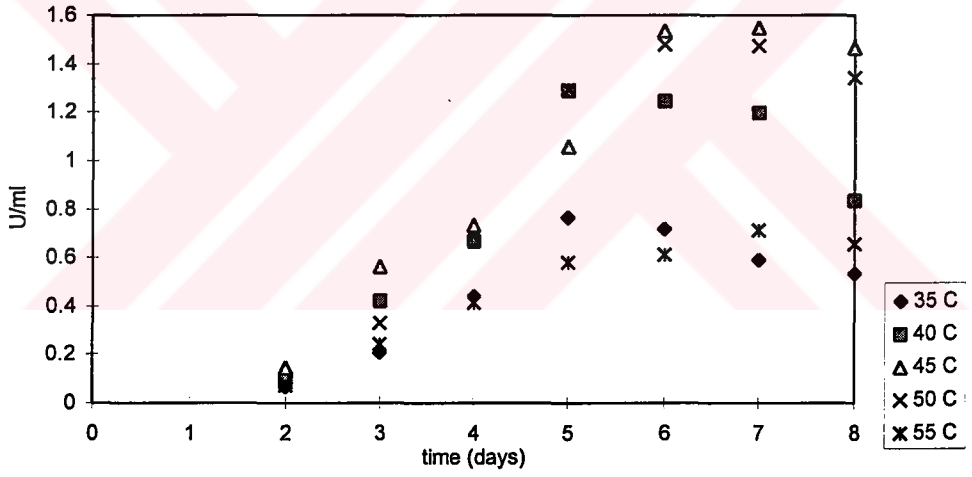


Fig. 3.9 Time course of EG production by *H.insolens* at different growth temperatures.

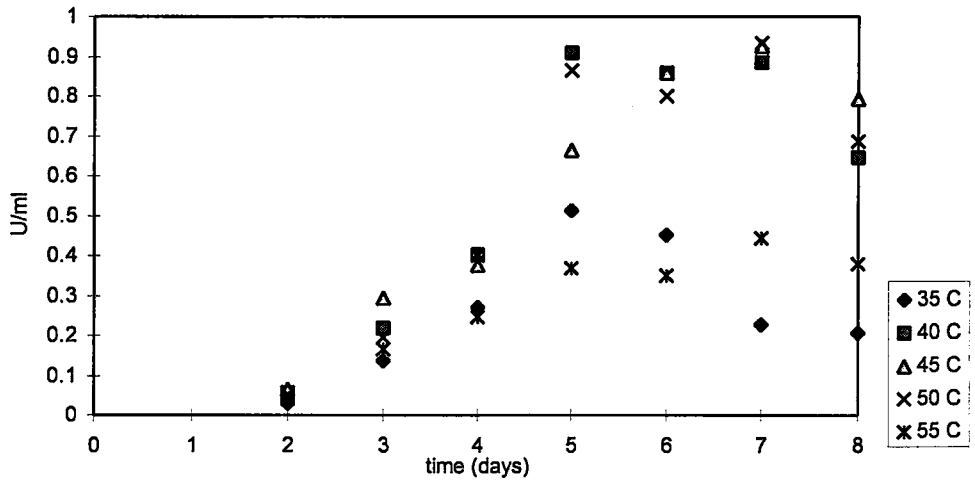


Fig. 3.10 Time course of AAEG production by *H.insolens* at different growth temperatures.

industrial production of these cellulases, because in industrial fermentations it is difficult to keep the temperature constant and uniform throughout the bioreactor. This is essentially the case for solid-substrate cultivations (Atev *et al.*, 1987).

For both *T.thermophila* and *H.insolens*, in general, after the 7th day of incubation, a decrease in the enzymatic activities has been detected. Only for FPA of *T.thermophila* at 40°C, there was an increase up to the 8th day of cultivation. The decrease in the cellulolytic activities might be due to the presence of proteases in the culture media, which could inactivate cellulases by proteolytic degradation. Hayashida and Mo (1986) have obtained a protease-negative *H.grisea* var. *thermoidea* and studied its cellulase production. The results have revealed that among the tested mutants, the protease-negative mutant produced the highest FPA, EG and AAEG activities, while the high-protease mutant produced no FPA and AAEG, and the lowest EG activity.

3.4.2 Effect of Temperature on the Growth of *T.thermophila* and *H.insolens*

Besides cellulase production, fungal biomass generation and cellulose utilization (based on the amount of insoluble cellulose in the supernatant) were investigated during cultivations at different temperatures. For *T.thermophila* highest fungal biomass production was obtained at 45°C (Fig. 3.11). Cellulose utilization of *T.thermophila* was almost the same at 35 to 50°C (Fig. 3.12). For *H.insolens*, maximal fungal biomass was generated at 45°C (Fig. 3.13). Avicel (cellulose) was maximally utilized at 45–50°C (Fig. 3.14).

While considering the cellulase production, fungal biomass generation and cellulose utilisation, it might be concluded that for *T.thermophila* and *H.insolens*, the temperature range of 40–50°C was favourable for fungal growth and cellulolytic activities.

The sporulation of the fungi when grown in the modified YpSs medium was visually examined. It appears that the time of sporulation is effected by growth temperature. At 35°C sporulation began at the fourth day, at 40°C – at the fifth, at 45°C – at the sixth, at 50°C – at the seventh days of incubation, while at 55°C no sporulation was observed for both organisms. At cultivation temperature of 55°C, for both *T.thermophila* and *H.insolens*, no germination of the spores in the preculture was observed, though the germinated fungi were able to grow at 55°C, when a preculture at 50°C was prepared. The sporulation and germination of any organism are dependent on temperature and the temperature range of sporulation and germination is usually narrower than that which allows growth (Carlile and Watkinson, 1995).

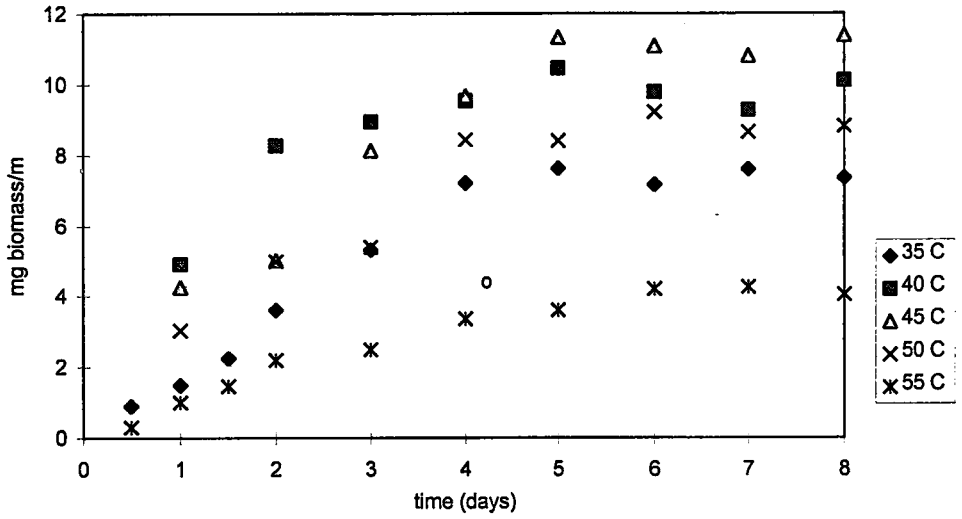


Fig. 3.11 Time course of fungal biomass generation of *T.thermophila* at different growth temperatures

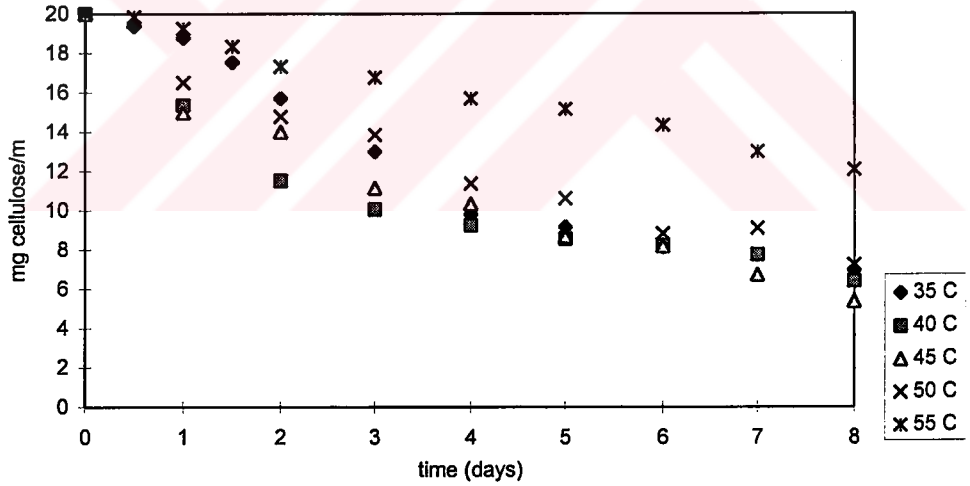


Fig 3.12 Time course of cellulose utilization by *T.thermophila* at different growth temperatures

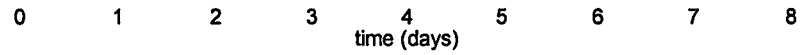


Fig 3.13 Time course of fungal biomass generation of *H.insolens* at different growth temperatures

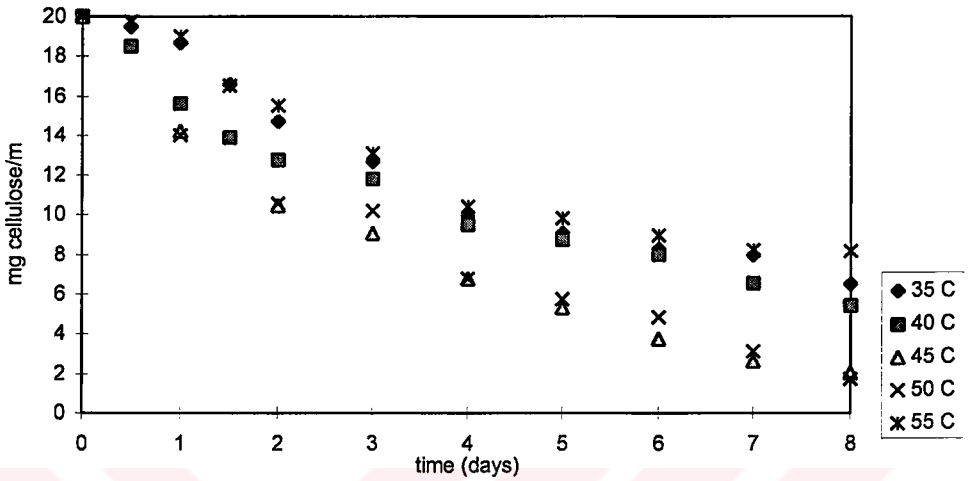


Fig. 3.14 Time course of cellulose utilization by *H.insolens* at different growth temperatures

3.4.3 Comparison of the Cellulase Production of *T.thermophila* and *H.insolens*

When the maximum cellulolytic activities of *T.thermophila* and *H.insolens*
grown at different temperatures are plotted in terms of U/ml (Fig. 3.15–3.17) and U/g biomass (Fig. 3.18–3.20) a number of observations are made. First, it appears that the FPA activities of *H.insolens* are higher than *T.thermophila*, especially at temperatures above 45°C (Fig. 3.15). From Fig. 3.17, it was found out that *T.thermophila* produced slightly higher AAEG than *H.insolens* at 45 and 50°C. *H.insolens* gave slightly higher EG activity (Fig. 3.16) than *T.thermophila*, which may seem to be negligible. However, on an industrial scale such small differences might be considerable when considering fermentors of several tones capacity. Nevertheless, such slight differences in the results could as well be due to experimental errors

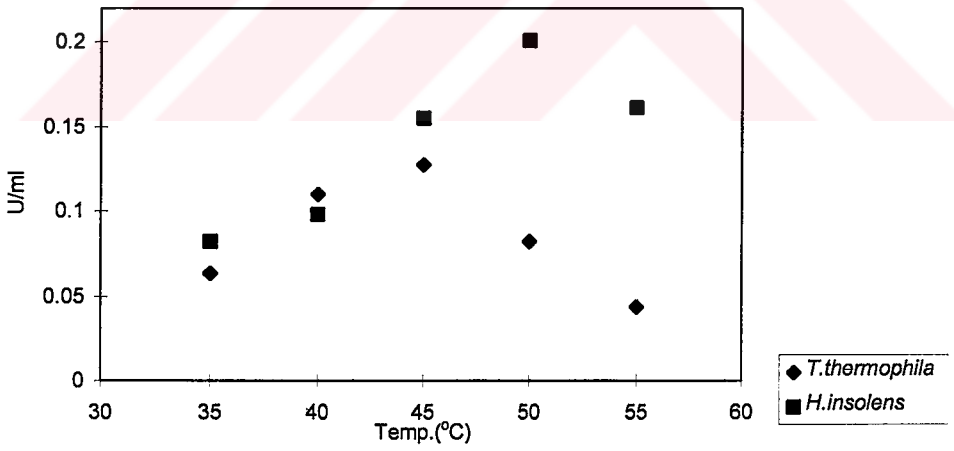


Fig. 3.15 Effect of growth temperature on the production of FPA by *T. thermophila* and *H.insolens* (Enzyme activity in terms of U/ml)

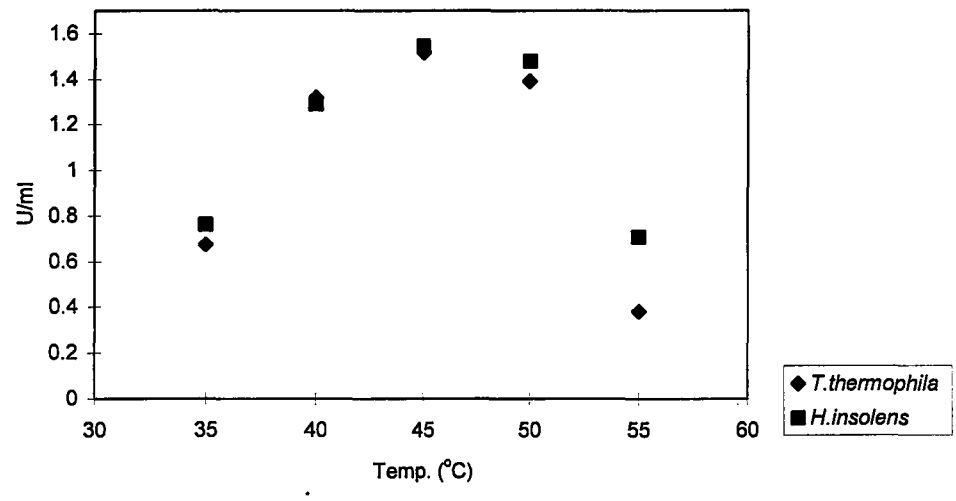


Fig. 3.16 Effect of growth temperature on the production of EG by *T. thermophila* and *H.insolens* (Enzyme activity in terms of U/ml)

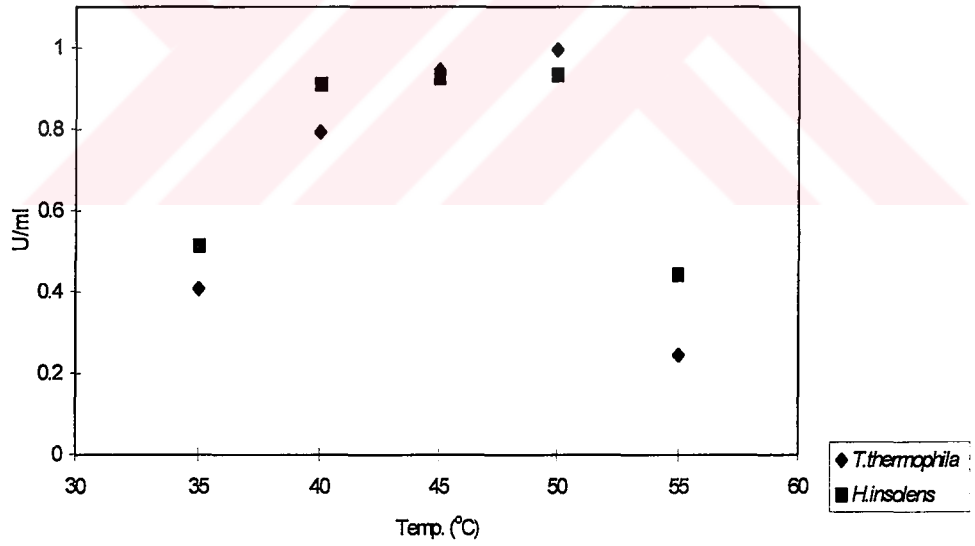


Fig. 3.17 Effect of growth temperature on the production of AAEG by *T.thermophila* and *H.insolens* (Enzyme activity in terms of U/ml)

The results in Fig. 3.18–3.20 are not so consistent with those in Fig. 3.15–3.17. The maximum FPA per gram fungal biomass of *T.thermophila* was obtained at 45°C (Fig. 3.18), as is the case for FPA activity in terms of U/ml (Fig. 3.15). For FPA per gram fungal biomass of *H.insolens*, a maximum value is not reached, since the enzyme activity continues to increase in the temperature range tested (Fig. 3.18). The EG activity of *T.thermophila* per gram biomass is somewhat higher than that of *H.insolens*. For EG and AAEG activities per gram fungal biomass, the maximum values for both *T.thermophila* and *H.insolens* were obtained at 50°C (Fig 3.19 and 3.20). A drop in the EG and AAEG activities per gram fungal biomass for both fungi was observed at 45°C, which can be explained by the high fungal biomass generation at this temperature. It can be concluded that the newly isolated strain of *T.thermophila* is as good EG and AAEG producer as the industrial strain of *H.insolens*, though FPA of *H.insolens* was considerably higher than that of *T.thermophila* under the provided growth conditions.

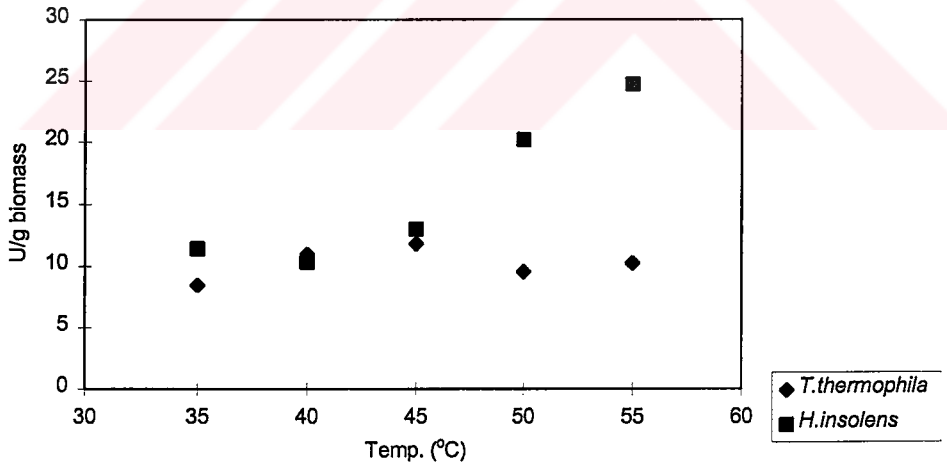


Fig. 3.18 Effect of growth temperature on the production of FPA per gram fungal biomass of *T.thermophila* and *H.insolens*

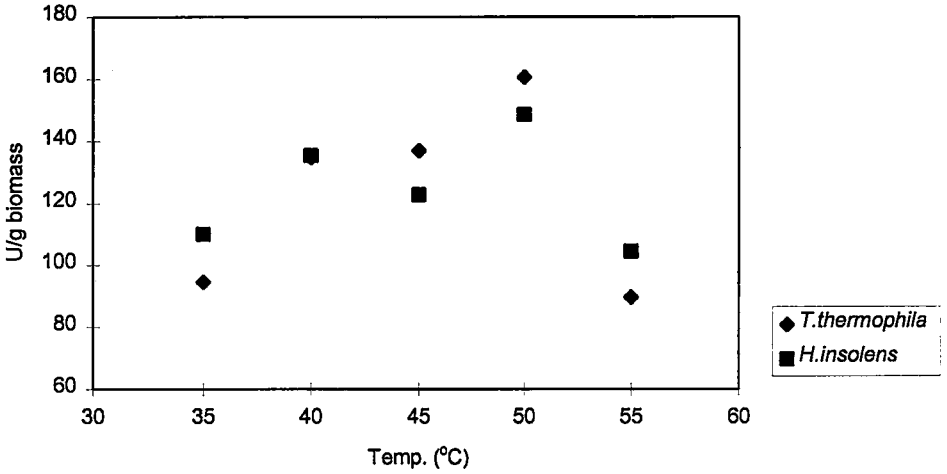


Fig. 3.19 Effect of growth temperature on the production of EG per gram fungal biomass of *T.thermophila* and *H.insolens*

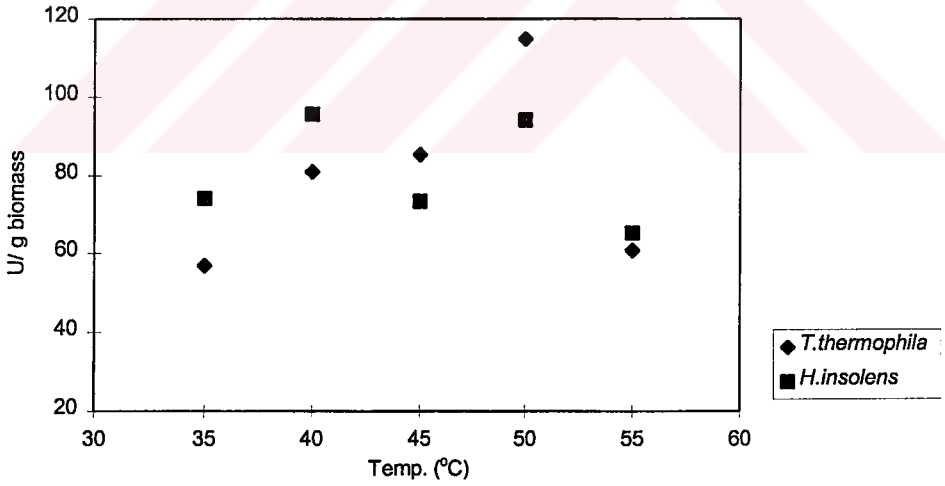


Fig. 3.20 Effect of growth temperature on the production of AAEG per gram fungal biomass of *T.thermophila* and *H.insolens*

The maximum EG and AAEG activities of *T.thermophila* and *H.insolens* at different temperatures are given in Table 3.2. From these results, it was found out that at 45°C, 62.3 % of the EG produced by *T.thermophila* was AAEG, while for *H.insolens* this value was 59.8 %. At 50°C, 71.6 % of the EG from *T.thermophila* was AAEG, while for *H.insolens* this value was 63.2 %. As it can be seen in Table 3.2, *T.thermophila* produced higher amounts of EG which could be adsorbed onto Avicel, and also the ratio of AAEG to EG was higher above 45°C for *T.thermophila.* This result might be of advantage for the industrial application of *T.thermophila* for the production of AAEG, since it indicates that a higher proportion of the total EG secreted has the ability of adsorption onto microcrystalline cellulose (Avicel). The structure of cotton also resembles that of microcrystalline cellulose, though there are also amorphous portions in cotton. Therefore, this enzyme might be readily used in textile industry since in finishing of clothes the pH is desired to be around neutrality and the optimum pH of the EG from *T.thermophila* is suitable for this purpose (Sec. 3.2).

Table 3.2 Maximum EG and AAEG activities of *T.thermophila* and *H.insolens* (Enzyme activities in terms of U/ml)

	T.thermophila			*H.insolens*		
	EG	AAEG	% AAEG	EG	AAEG	% AAEG
35°C	0.6755	0.407	60.3	0.762	0.512	67.2
40°C	1.317	0.791	60	1.289	0.91	70.6
45°C	1.5165	0.945	62.3	1.547	0.925	59.8
50°C	1.389	0.9945	71.6	1.475	0.9325	63.2
55°C	0.3805	0.2335	61.4	0.708	0.4415	62.4

3.4.4 Comparison of the Specific Activities of the Cellulases of *T.thermophila and H.insolens*

The extracellular protein excreted into culture media was measured for both fungi. Again, for both *T.thermophila* (Fig 3.21) and *H.insolens* (Fig. 3.22) the temperature range of 40 to 50°C seemed to yield highest protein production, with a maximum at 45°C for *T.thermophila*. The extracellular protein at 35 and 55°C after the fifth day of incubation for both organisms was almost the same.

The maximum protein produced at different temperatures for *T.thermophila* and *H.insolens* are given in Fig. 3.23. *H.insolens* produced higher protein than *T.thermophila*. *T.thermophila* produced maximum exocell protein at 45°. While for *H.insolens* a temperature range of 40–50°C favored protein production, the maximum value being at 50°C.

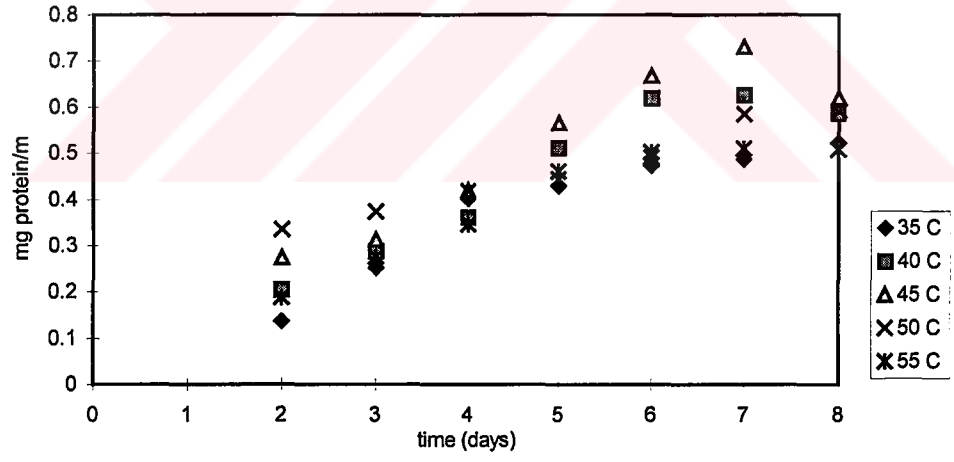


Fig. 3.21 Time course of extracellular protein production by *T. thermophila* at different growth temperatures

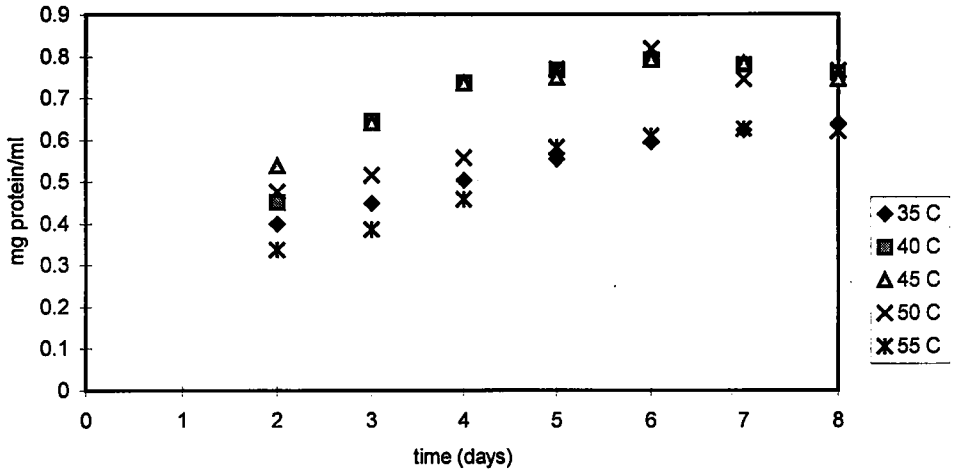


Fig. 3.22 Time course of extracellular protein production by *H.insolens* at different growth temperatures

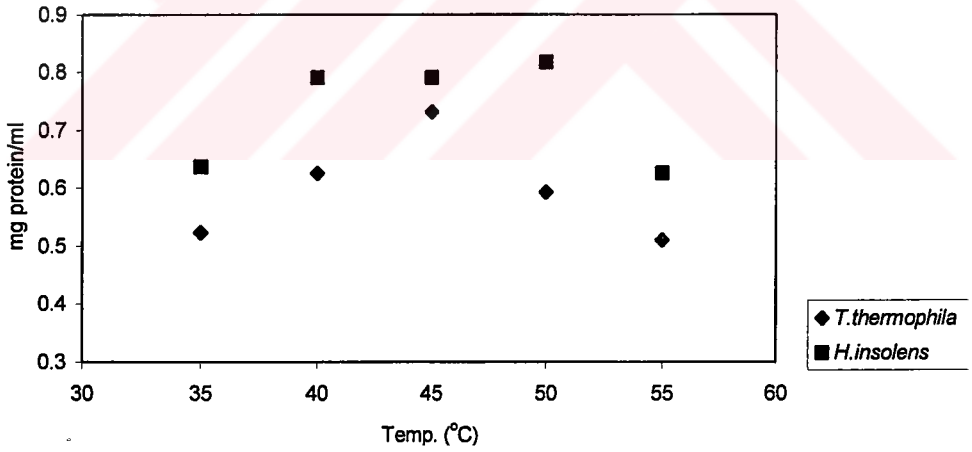


Fig. 3.23 Effect of growth temperature on the production of extracellular protein by *T.thermophila* and *H.insolens*

The specific activities of FPA, EG and AAEG at 40, 45 and 50°C are given in Table 3.3. Accordingly, the specific FPA activity of *H.insolens* is markedly higher at 50°C than that of *T.thermophila*. On the other hand, the specific EG and AAEG from *T.thermophila* at 40, 45 and 50°C were higher than those of *H.insolens*. The fact that specific EG and AAEG activities of *T.thermophila* are higher, might be of great advantage, in terms of the purification of these enzymes, since the higher the specific activity the less is the amount of the secreted proteins that should be removed. In general, industrial enzymes are not entirely pure. Some of the impurities originate from the culture medium whereas others are deliberately added as bulking agents. However, the purer the enzyme the better, as it is likely that contaminants cause unwanted side reactions in industrial processes.

Table 3.3 Specific activities of the cellulolytic enzymes of *T.thermophila* and *H.insolens*

		40°C	45°C	50°C
Filter Paper Activity (U/mg protein)	*T.thermophila*	0.188	0.174	0.14
	H.insolens	0.133	0.196	0.245
EG Activity (U/mg protein)	*T.thermophila*	2.129	2.27	2.374
	H.insolens	1.678	1.978	1.98
AAEG Activity (U/mg protein)	*T.thermophila*	1.279	1.415	1.7
	H.insolens	1.185	1.183	1.252

3.4.5 Changes in the Medium pH during Cultivation of *T.thermophila* and *H.insolens*

The changes in the medium pH during cultivation of *T.thermophila* and *H.insolens* are given in Fig 3.24 and 3.25, respectively. For *T.thermophila*, the medium pH changed from 6.24 to 8.37 (Fig. 3.24), while for *H.insolens* the medium pH varied in the range of 5.75–7.55 (Fig 3.25), with an initial value of 7.5 for both organisms. It could be said that these changes in the medium pH were not so sharp and might not affect the enzymatic activities negatively. The changes in the medium pH might be due to the metabolic activities of the organisms during growth.

Since the organisms were grown in shake flasks, the medium pH could not be kept constant. In the fermentation medium, there were salts such as K_2HPO_4 which might have had a buffering effect on the medium pH, since the changes in the pH were not so sharp.

In studies on the production of cellulases, the effect of pH on the cellulase synthesis was investigated. *T.reesei* QMY-1 was grown on pure cellulose at different pH values ranging between 3.0 and 7.0. A pH level of 5.0 and 6.0 appeared to be optimal for FPA production (Chahal *et al.*, 1992).

In future studies on the cellulase production by *T.thermophila* and *H.insolens*, these organisms might be grown in fermentors and the effect of medium pH might be investigated.

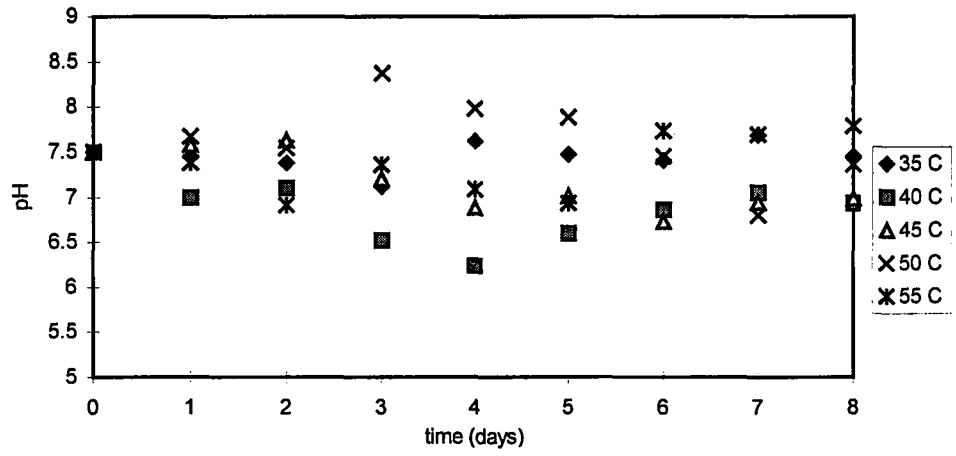


Fig. 3.24 Changes in medium pH during cultivation of *T.thermophila* at different temperatures

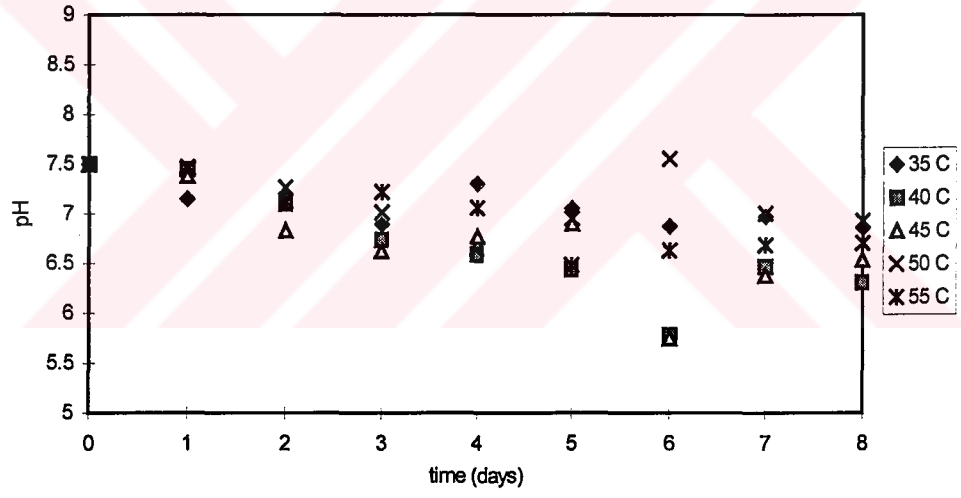


Fig. 3.25 Changes in medium pH during cultivation of *H.insolens* at different growth temperatures

3.5 Comparison of the EG Activities of *T.thermophila* and *H.insolens* with Industrial Cellulase Preparations

The specific EG activities in culture supernatants of *T.thermophila* and *H.insolens*, grown under laboratory conditions were compared with EG activities in industrial cellulase preparations of Novo Nordisk and of ORBA Inc. (Sec. 2.2.2.2). As shown in Table 3.4, the specific activities of the EGs from *T.thermophila* and *H.insolens* grown at 45°C under laboratory conditions were higher than those of EGs of Novo Nordisk and ORBA.

From the results in Table 3.4, it was found out that the specific EG activity in *T.thermophila* culture supernatants at 45°C is 1.4 times higher than the commercial enzyme preparation of Novo Nordisk, and 10.6 times higher than the ORBA enzyme preparations. As for *H.insolens* grown at 45°C, 1.2 times higher specific EG activity was obtained, compared to Novo Nordisk enzyme preparation, and 9.3 times higher compared to the ORBA preparation. These were the results when specific activity was considered. To make a comparison of activities in terms of U/ml of fermentation medium was not possible, since the necessary data were not available for the industrial cellulase preparations.

When comparing enzyme activities, the activity in terms of U/ml of culture supernatant gives knowledge about the volume required to be used to obtain the same amounts of enzyme. In industrial fermentations, the volume of the process tanks required for the cultivations is of importance since with increase in volume, some problems might arise such as difficulties in agitation, keeping uniform cultural conditions and so on. The activity in terms of U/g protein gives knowledge about how much of the protein produced by a specific organism is composed of the desired enzyme. This is important for the downstream processing of the enzyme.

The cellulase preparation of ORBA Inc. is obtained from solid state fermentation, while there is no information about the cellulase of Novo Nordisk. At least the enzyme preparation of ORBA Inc. is known to be obtained by using the entire mixture of the solid fermentation medium. This can explain the lower specific activities due to a higher total protein content. Besides this, the experiments in our laboratory were carried in shake flasks and C-source used was pure cellulose (Avicel) which is quite effective in inducing cellulase synthesis. Avicel is however not used as the whole C-source in industrial fermentations because of its high cost. Instead, in most applications, agricultural and industrial by-products containing cellulose are used as the industrial raw materials. Such raw materials often yield lower activities (Atev *et al.*, 1987).

Table 3.4 Comparison of the EG specific activities obtained in this study with those of industrial preparations

EG of *T.thermophila* Grown in lab. conditions (U/mg protein)	EG of *H.insolens* grown in lab. conditions (U/mg protein)	EG (Novo Nordisk) (U/mg protein)	EG (ORBA) (U/mg protein)
2,27	1,978	1,604	0,214

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

In this study, the cellulase activity and growth of *T.thermophila* and *H. insolens* were analyzed and compared. A suitable medium for cellulase production was selected. The EG, Avicel-adsorbable EG and total cellulase (FPA), as well as extracellular protein, cellulose utilisation, fungal biomass generation and medium pH have been measured during the cultivation of the fungi.

The modified YpSs medium was found to be suitable for EG and total cellulase production for both *T.thermophila* and *H.insolens*, since in this medium the fungi produced much higher FPA and EG activity than in the modified Czapek's medium.

The effect of assay pH and temperature on the activity of EG of *T. thermophila* and *H.insolens* have been investigated. For the EG of *H.insolens*, the optimum assay conditions were found to be pH 6.5 and 60°C, while for *T. thermophila* EG, best conditions were at pH 6.0 and 65°C.

The study on the effect of temperature on the growth and cellulase production by the fungi have shown that both *T.thermophila* and *H.insolens* might grow and produce cellulolytic enzymes in the range of 35-55°C, and for both organisms a temperature range of 40-50°C was favourable for growth and cellulase production. For both *T.thermophila* and *H.insolens*, the maximal EG

(U/ml) were obtained at 45°C. Though these values with those at 50°C were quite close to each other. Again, maximal fungal biomass was generated at 45°C, the values being quite close to those obtained at 40 and 50°C.

A comparison of the cellulase production of the fungi has revealed that the newly isolated strain of *T.thermophila* was as good EG and AAEG producer as the industrial strain of *H.insolens* under the given fermentation conditions, while the total cellulase activity (FPA) of *H.insolens* was considerably higher than that of *T.thermophila*.

The EG specific activities of *T.thermophila* and *H.insolens* grown under laboratory conditions were compared with those of the industrial cellulase preparations of Novo Nordisk and ORBA. It was found out that the specific activity of EG from *T.thermophila* and *H.insolens* grown in laboratory conditions were higher than those of the industrial preparations.

To optimize the cellulase production from *T.thermophila* and *H.insolens*, some further studies on the cultivation of the fungi in fermentors might be done. Especially, the effect of pH of the fermentation medium on cellulase production might be studied. Further, the cellulase enzymes might be purified and their properties might be investigated. The thermal and pH stabilities of the cellulases from *T.thermophila* and *H.insolens* might be analyzed. Some agricultural waste products might be used as carbon and nitrogen sources in order to see their effects on the production of cellulases by *T.thermophila* and *H.insolens*.

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APPENDIX A

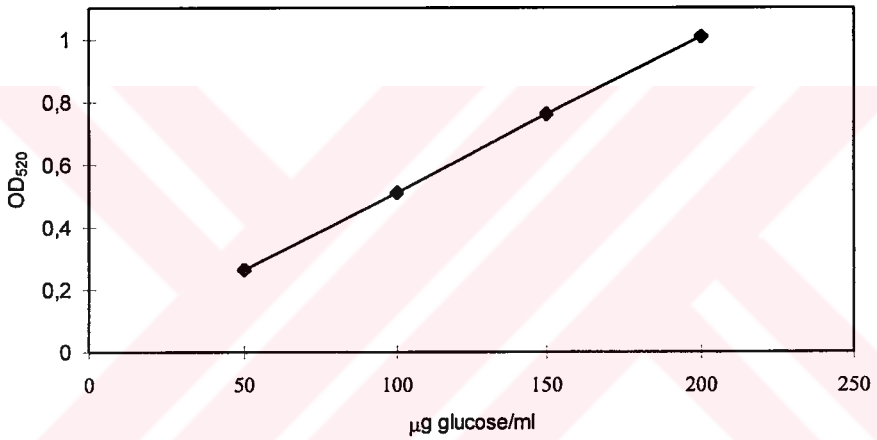

Fig. A.1 Standard curve for Somogyi-Nelson method for determination of reducing sugar

APPENDIX B

COMPOSITIONS OF SOMOGYI I, SOMOGYI II AND NELSON REAGENTS

Somogyi Reagent I:

288 g of Na_2CO_3 (anhydrous) are dissolved in 1 liter boiled distilled water. 24 g of $C_4H_4KNaO_6.4H_2O$, 48 g of Na_2CO_3, and 32 g of $NaHCO_3$ are added, and the solution is diluted to 1600 ml with boiled distilled water. The solution is stored at 27°C.

Somogyi Reagent II:

72 g of Na_2SO_4 are dissolved in 300 ml boiled distilled water. 8 g of $CuSO_4$ are added, and the solution is distilled to 400 ml with boiled distilled water. The prepared solution is stored at 27°C.

Immediately before use, Somogyi reagent I and II are mixed in 4:1 ratio.

Nelson Reagent:

100 g of $(NH_4)_6Mo_7O_{24}.4H_2O$ is dissolved in 1.8 liters of distilled water and 84 ml of concentrated H_2SO_4 is added. Further, 100 ml of $Na_2HAsO_4.7H_2O$ solution is added (12 g of $Na_2HAsO_4.7H_2O$ is dissolved in 100 ml of distilled water). The prepared solution is placed in brown glass bottle and stored at 37°C for 24-48 hr and then at room temperature.

APPENDIX C

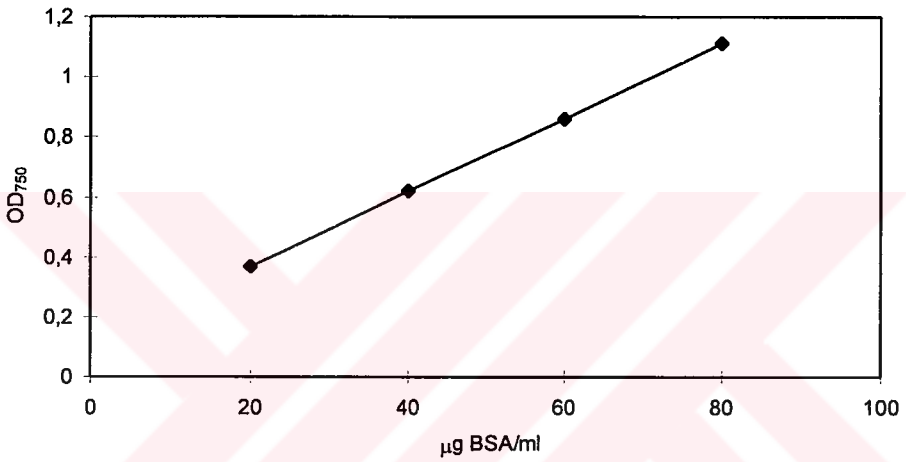

Fig. C.1 Standard curve for Lowry's method for protein determination

APPENDIX D

COMPOSITIONS OF REAGENTS A, B, C AND D

Reagent A:

2.0 g of NaOH, 10.0 g of Na_2CO_3 and 0.1 g of $C_4H_4KNaO_6.4H_2O$ are dissolved in 500 ml of distilled water.

Reagent B:

0.5 g of $CuSO_4.5H_2O$ are dissolved in 100 ml of distilled water.

Reagent C:

10 ml of Reagent A and 0.2 ml of Reagent B are mixed immediately before use.

Reagent D:

Folin phenol (2 N) is mixed with distilled water in 1:1 ratio immediately before use.

APPENDIX E

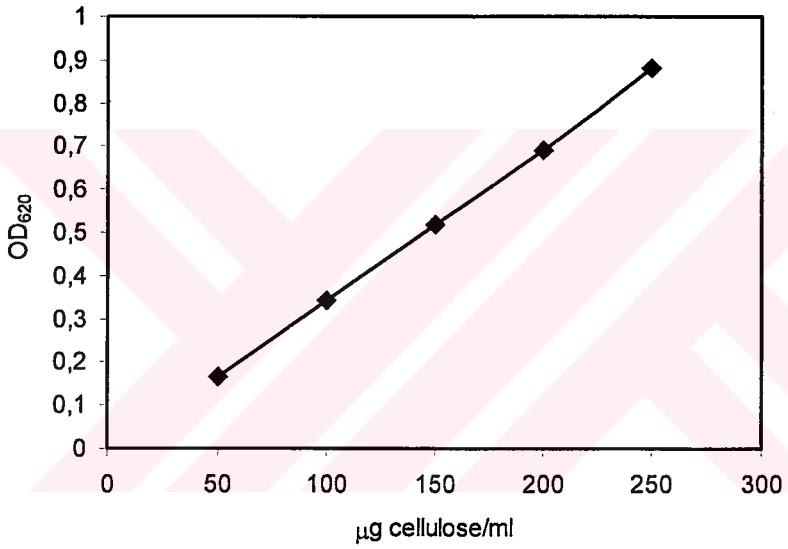

Fig. E.1 Standard curve for Uppdegraff's method for cellulose determination

APPENDIX F

COMPOSITIONS OF ACETIC-NITRIC AND ANTHRONE REAGENTS

Acetic-Nitric Reagent:

150 ml of 80 % acetic acid is mixed with 15 ml of concentrated HNO_3.

Anthrone Reagent:

0.2 g of anthrone is added to 100 ml of concentrated H_2SO_4. The reagent is prepared fresh daily, and is chilled about 2 hr in refrigerator prior to use.